DNA DEGRADING ENZYMES OF THE SLIME MOLD PHYSARUM POLYCEPHALUM SCHWEIN.

B.J.J. POLMAN

Errata:

- Page 28 should be read as page 29 and vice versa
- Page 52 should be read as page 53 and vice versa
- Page 66, figure 33: NE: nuclear envelope

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Promotor: Prof. Dr. Ch. M. A. KUYPER

DNA DEGRADING ENZYMES OF THE SLIME MOLD PHYSARUM POLYCEPHALUM SCHWEIN.

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
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VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
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Druk: Stichting Studentenpers Nijmegen

Aan mijn ouders,

Voor An, Hanna en Pieter

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Abbreviations and symbols

AMP	adenosine-5'-monophosphate
Ci	Curie
CM	carboxymethyl
CMP	cytidine-5'-monophosphate
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpm	desintegrations per minute
EC	Report of the Commission on Enzymes of the International Union of Biochemistry, 1961
EDTA	ethylenediaminetetra acetate
GMP	guanosine-5'-monophosphate
^{3}H-UTP	tritiated uridine triphosphate
min.	minutes
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
Schwein.	Schweinitz, L.D. von
SDS	sodium dodecylsulfate
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tris	tris'(hydroxymethyl)-aminomethane
UTP	uridine-5'-triphosphate

λ	wave length
ΔE_{260}	increase of absorbance at 260 nm
3.2 - N	DNA degrading activity on native DNA at pH 3.2
3.2 - D	DNA degrading activity on denaturated DNA at pH 3.2
4.0	" " " " " " at pH 4.0
7.6	" " " " " " at pH 7.6
8.5	" " " " " " at pH 8.5

CHAPTER I

GENERAL INTRODUCTION

The nucleases are a special class of the phosphodiesterases. They are able to depolymerize ribonucleic and deoxyribonucleic acids (Laskowski, 1967). The nucleases which degrade principally deoxyribonucleic acid are called deoxyribonucleases (Laskowski, 1967; Schmidt, 1970). They are found in many organisms (Laskowski, 1961, 1967; Lesca, 1971) and in most organisms more than one deoxyribonuclease is found (Laskowski, 1967; Lesca, 1971). Thus bacteria, e.g. Escherichia coli (Lehman, 1967) and mammalian tissues (Laskowski, 1967; Lesca, 1971) contain several deoxyribonucleases.

The deoxyribonucleases are thought to be related to many processes in the cell. They are supposed to play a role in the nucleic acid catabolism (Richardson, 1969; Koerner, 1970), DNA synthesis (Kornberg, 1964; Lehman, 1967), DNA transcription (Vogt, 1969), genetic recombination (Buttin and Wright, 1968; Oishi, 1969; Winder, 1972), DNA repair (Friedman and Goldwaith, 1968; Takagi et al., 1968; Setlow and Carrier, 1973), cell differentiation (Boyd and Boyd, 1970; De Petrocellis and Parisi, 1972), the defence against strange DNA (e.g. restriction) (Herriott et al., 1961; Zahn and Kleinschmidt, 1961; Gosse et al., 1965; Arber and Linn, 1969) and the digestion of food (Schmidt, 1970).

Apart from trying to get more insight in the different cellular processes, deoxyribonucleases are also studied for one specific other reason, namely to detect and isolate deoxyribonucleases which attack deoxyribonucleic acid in a specific way at specific internucleotide bonds, so that these enzymes can be used as proper tools to elucidate the deoxyribonucleic acid primary structure of many organisms (Privat de Garilhe, 1964; Laskowski, 1967; Bernardi et al., 1973).

A very attractive organism to study the regulation of many cellular processes is the slime mold Physarum polycephalum. This slime mold exhibits natural synchrony of nuclear division and can undergo synchronous differentiation: sclerotization, or sporulation (Guttes et al., 1961). It has been used for many studies concerning the synthesis of DNA (Rusch, 1969; Schiebel, 1973; Werry, 1973), of RNA (Chet, 1973) and of enzymes (Hüttermann et al., 1970; Hüttermann,

1973; Schiebel, 1973), for studies concerning the regulation of mitosis (Rusch et al., 1966; Oppenheim and Katzir, 1971; Schiebel, 1973) and differentiation (Chet, 1973; Hüttermann, 1973; Sauer, 1973). The genetic analysis of this slime mold has reached an advanced stage (Dee, 1973).

However, hardly anything is known about the deoxyribonucleases of Physarum polycephalum. Only Hiramaru et al. (1969) mentioned the existence of a deoxyribonuclease activity in crude enzyme extracts of the slime mold with maximum activity at pH 4.5 and they could isolate two non-specific nucleases which were able to degrade denaturated DNA as well as RNA. More work has been carried out on the ribonucleases of the slime mold. A survey of the literature concerning the nucleases of Physarum polycephalum is given in Tables I and II and, as one can see, several ribonucleases have been identified in the slime mold until now.

Heterophagy and intracellular digestion of engulfed food materials play a fundamental role in the myxamoebae, to which Physarum polycephalum belongs (Gray and Alexopoulos, 1968; Kazama and Aldrich, 1972) and in this slime mold pinocytosis of liquid drops has been observed (Guttes and Guttes, 1960). To digest the engulfed material a battery of hydrolytic enzymes is needed (Kazama and Aldrich, 1972). In some slime molds organelles containing strong acid phosphatase activity (Gizelius, 1971; Kazama and Aldrich, 1972) and in others lysosome-like particles have been observed (McManus, 1965; Kazama and Aldrich, 1972). Until now such particles have not been described in Physarum polycephalum, although extracts of this slime mold possess a high acid phosphatase activity (Hüttermann et al., 1970) and many other hydrolytic enzymes (Hüttermann, 1973). Probably several ribonucleases of Physarum polycephalum play a role in the digestion of food. We therefore could expect Physarum polycephalum to possess various DNA degrading enzymes as well.

Our first aim was to identify and characterize the DNA degrading enzymes of the slime mold (chapter IV and V). Secondly, we wanted to know if these enzymes are related to processes which regulate growth and differentiation in the slime mold (chapter VI). Thirdly, we studied DNase activities associated with nuclei isolated from the slime mold (chapter VIII). In chapter VII investigations on the isolation of nuclei of Physarum polycephalum are described.

Table I. Survey of the literature concerning the characteristics of the nucleases of the slime mold Physarum polycephalum.

enzyme	substrate	optimum pH	molecular weight	specificity for	products of hydrolysis	thermo-stability	intra, or extra cellular	ref.
RNase Pp-1*	RNA	6.7	40.000	guanosine	-3'-guanylic acid -2',3'-cyclic guanylic acid -oligonucleot.	stable	intra	1
RNase Pp-2	RNA	4.5	40.000	non-spec.*	all mononucleotides	stable	intra	1
RNase Pp-3	RNA	5.5	10.000	prefers purine nucleoside bonds	no pyrimidine mononucleotides	stable	intra	1
RNase Pp-4	RNA	4.0	n.d.*	non-spec.	-5'-P terminal -3'-P-purine terminal	labile	intra	2
Nuclease Pp-1	RNA and single stranded DNA	4.5	n.d.	non-spec.	-3'-P terminal	labile	intra	2
Nuclease Pp-2	RNA and single stranded DNA	4.5	40.000 - 100.000	non-spec.	-3' and 5'-P terminal	labile	intra	2
RNase I	RNA	4.0	31.000	-dislikes cytosine bonds	-oligonucl. -3'-A,U,GMP -2',3'-CMP	labile	extra and intra	3
RNase	n.d.	4.5 - 5.0	n.d.	n.d.	n.d.	labile	extra and intra	4

* n.d.: not-described; non-spec.: non-specific; Pp: Physarum polycephalum.

References: 1. Hiramaru et al., 1969a; 2. Hiramaru et al., 1969b; 3. Braun and Behrens, 1969; 4. Farr et al., 1972.

Table II. Survey of the literature concerning the behaviour of the nucleases and phosphodiesterases of Physarum polycephalum during growth and differentiation.

enzyme	mitotic cycle	specific activity during sperulation	references
RNase I	-stepwise doubling during 3 - 4 hours after mitosis		1
RNase		-increase during first 2 hours, then decrease*	2
RNase		-fourfold increase during the first 6 hours after mitosis, then decrease to the original value at 12 h, unchanged after this time	3
Phosphodiesterase I	-continuous increase	-eighteenfold increase -twofold increase*	3,4 3
Phosphodiesterase II		-not detectable after 12 h -twofold increase*	3 3

* spherulation due to mannitol addition

References: 1. Braun and Behrens, 1969; 2. Chet et al., 1973; 3. Hüttermann, 1973; 4. Hüttermann et al., 1970.

CHAPTER II

MATERIAL AND GENERAL METHODS

A. The organism

The experiments were carried out with a line of Physarum polycephalum kindly supplied by Dr. W. Sachsenmaier (Innsbruck, Austria). The slime mold was cultured essentially according to Daniel and Baldwin (1964). The culturing of microplasmodia and the preparation of macroplasmodia in the Laboratory of Chemical Cytology is described in detail by Werry (1973).

In the experiments described in the following chapters macroplasmodia were harvested between 16 and 25 hours after coalescence, washed with ice-cold aqua bidest. and frozen in liquid air. Mitosis III was observed between 18 and 22 hours after coalescence. The time of occurrence of the metaphase varied no more than 15 minutes between the several plasmodia.

Sperule formation was induced by harvesting microplasmodia from late logarithmic phase shaken cultures by low-speed centrifugation (500 g for 2 min.), washing once with 10 volumes of non-nutrient salt medium and shaking these flasks under the same conditions as growing cultures. The composition of the non-nutrient salt medium was the same as the citrate-hematin semi-defined growth medium, without tryptone, yeast extract and glucose. After about 24 hours the first hard-walled spherules were observed.

Spherule germination was induced by harvesting microplasmodia which had been starved on non-nutrient salt medium during 60 hours by low-speed centrifugation and resuspending them in 20 ml growth medium.

B. The preparation of crude enzyme extracts

Microplasmodia were collected by centrifugation for 2 min. at 500 g, washed twice with aqua bidest. and once with 10 mM Tris/HCl, pH 7.0 containing 10 mM KCl. Without KCl in the wash and homogenisation buffers lower and unreproducible enzyme activities were obtained. The washed microplasmodia were suspended in two volumes 10 mM Tris/HCl, pH 7.0 - 10 mM KCl - 0.1% Triton X-100 and these suspensions were treated with a MSE 150 Watt sonifier (6x10 sec.;

stage: low-4) followed by centrifugation at 1500 g during 10 min. The supernatants were used as the crude enzyme extract. The final pH of these extracts was 7.1. All steps were carried out at 0-4°C.

Later on, the washing step with wash buffer proved not to be necessary and was omitted (chapter V, VI and VII).

Samples of macroplasmodia or microplasmodia taken during the growth cycle, or from shaken cultures during growth and sporulation were washed, frozen in liquid air and stored at -20°C. Crude enzyme extracts from these samples were prepared as described above.

C. The determination of the DNA degrading activities

For measuring the DNase activity native or heat-denatured salmon sperm DNA (Sigma) was used as substrate. Denaturation of the DNA was achieved by heating the DNA solution (5 mg per ml aqua bidest.) at 95°C during 15 min. and then quickly cooling in an ice-water mixture. The incubation mixture consisted of 1.0 ml of an appropriate 0.3 M buffer (see below), 0.2 ml crude enzyme extract, 0.1 ml aqua bidest. at the acid pH's or 0.1 ml 75 mM or 15 mM $MgCl_2$ (at the alkaline pH's, see below) and 0.2 ml salmon sperm DNA (5 mg per ml aqua bidest.). After incubation at 30°C during 20 min. for the acid DNase activities (pH 2.5 - 6.0), or during 120 min. for the alkaline DNase activities (pH 6 - 9.5) the test tubes were chilled at 0°C during 2 min.; 0.1 ml Bovine serum albumin (Sigma, Fraction V powder, 50 mg/ml aqua bidest.) and 1.0 ml 2.5 N $HClO_4$ were added and the tubes were shaken vigorously. After 10 min. at 0°C the tubes were clarified by centrifugation for 10 min. at 3000 g and the absorbance of the supernatants was measured at 260 nm after appropriate dilution with aqua bidest.

Routinely DNase activity on native DNA was measured in 0.3 M sodium formate/formic acid buffer, pH 3.2. DNase activity on denaturated DNA at pH 3.2 and 4.0 was measured in 0.3 M sodium formate/formic acid buffer and at pH 7.6 and 8.5 in 0.3 M Tris/HCl. The reaction mixture at pH 7.6 contained 5 mM $MgCl_2$ and at pH 8.5 1 mM $MgCl_2$.

The absorbances at 260 nm were corrected for blank values, which were obtained by adding the DNA after incubation at 30°C and chilling the test tube at 0°C. Routinely all incubations and all blanks

were carried out in triplicate. The enzyme activity was expressed as Δ E260/mg protein/min.

Figure 1 presents the absorption spectrum of an incubated sample and a blank. As one can see enzyme activity was expressed as an increase of the absorption at 260 nm. The yellow pigment of Physarum polycephalum which has under these conditions an absorption maximum at 380 nm did not affect the absorption at 260 nm, because the absorption of the blanks was subtracted from the absorption of the incubated samples.

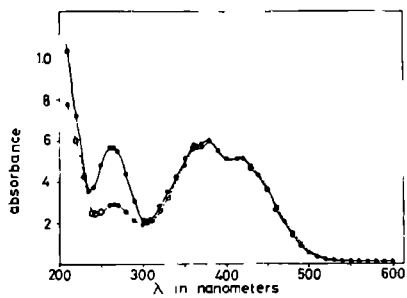


Figure 1. Absorption spectrum of an incubated sample and a blank after pelletting the macroplasmodial material with $HClO_4$.
● incubated sample, O blank.

Fig. 2 and 3 show that the amount of acid-soluble products increases linearly with the amount of extract until about a protein concentration of 1000 µg per incubation mixture. Care was taken that measurements were carried out with 100 - 700 µg protein in the incubation mixture.

At acid pH's the formation of acid-soluble products increased linearly during the incubation until about 30 minutes (figure 4). Especially at pH 3.2 this was not due to exhaustion of the substrate, but to breakdown of the enzyme molecules under these incubation conditions (see chapter IV). At alkaline pH's the DNA degrading activities were still linearly proportional to the incubation time after 6 hours (figure 5).

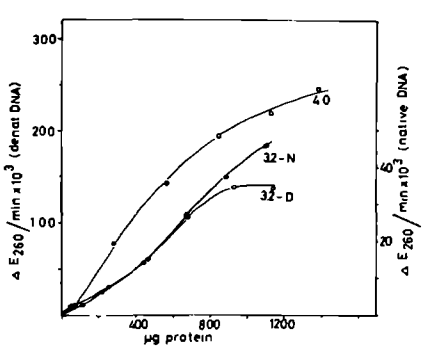


Fig. 2

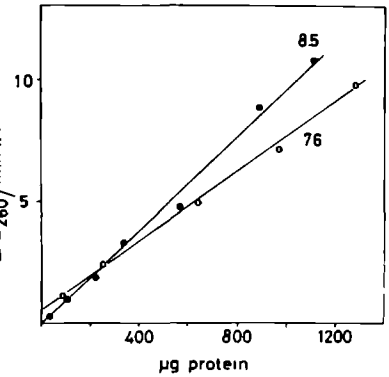


Fig. 3

Figure 2. Formation of acid-soluble products as a function of the concentration of the homogenate incubated at an acid pH.

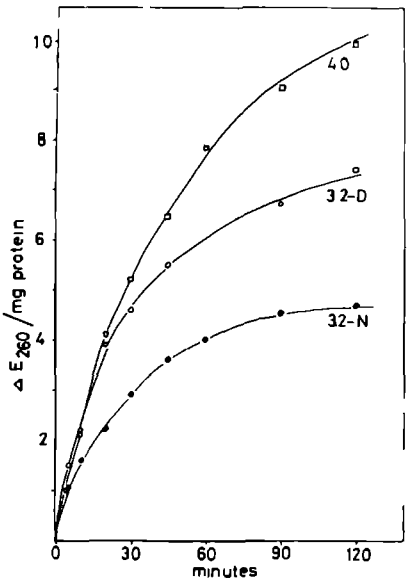
Figure 3. Formation of acid-soluble products as a function of the concentration of the crude enzyme extract incubated at an alkaline pH.

D. Determination of the RNA degrading activity

RNA degrading activity was measured according to Shortman (1961). The incubation mixture consisted of 1.0 ml 0.3 M buffer, 0.2 ml yeast RNA (10 mg/ml aqua bidest. for the pH-gradient experiments, 28 mg/ml aqua bidest. for the cell fractionation experiments), 0.2 ml crude enzyme extract and 0.1 ml aqua bidest. After incubation during 20 min. (in the acid region) or 60 min. (in the alkaline region) at 30°C the tubes were cooled to 0°C, 1.5 ml 1 N HCl in 76% ethanol was added and the tubes were shaken vigorously. After 10 min. at 0°C the tubes were clarified by centrifugation for 10 min. at 1500 g and the absorbance of the supernatants was measured at 260 nm. The absorbances at 260 nm were corrected for blank values, which were obtained by adding the RNA substrate after the incubation at 30°C and chilling the test tube at 0°C.

Purified yeast RNA (Schwarz) was used as substrate. Purification was as follows: 1% yeast RNA in aqua bidest. was precipitated with 1 volume 1 N HCl in 76% ethanol and washed twice with HCl-alcohol. The pellet was washed three times with 0.25% $HClO_4$ and dissolved in

aqua bidest. by adding of KOH until pH 7.0. The solution was dialyzed against 20 volumes 0.01 M EDTA (Titriplex III) and against 0.15 M NaCl with two changes, then against aqua bidest. with five changes of the dialyzing medium. The solution was made 1% and brought to pH 7, centrifuged for 30 min. at 3000 g and stored under -20°C. The more concentrated (e.g. 2.8%) RNA solutions were obtained by lyophilization of the 1% RNA solution.



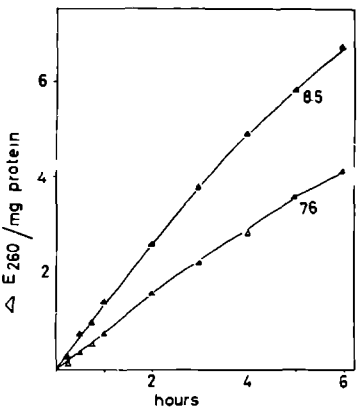


Fig. 4

Fig. 5

Figure 4. Formation of acid-soluble products at acid pH's as a function of the incubation time.

Figure 5. Formation of acid-soluble products at alkaline pH's as a function of the incubation time.

E. Determination of phosphatase and phosphodiesterase activity

Acid phosphatase activity was determined according to Hüttermann et al. (1970) with 4 - nitrophenyl - phosphate (Merck) as substrate.

To determine the effect of the pH on the phosphatase activity in the crude enzyme extract the same 0.3 M buffers were used as for

the DNA degrading activities. In the other experiments acid phosphatase was determined exactly as described by Hüttermann et al. (1970).

The determination of the phosphodiesterase activity in the crude enzyme extracts was adapted from Hüttermann et al. (1970). The incubation mixture contained 1.0 ml 0.3 M buffer (same buffers as were used for the DNA degrading enzymes), 0.2 ml 5 mM 4-nitrophenyl-thymidine-3' (or 5') phosphate (Sigma) in aqua bidest., 0.2 ml crude enzyme extract and 0.1 ml aqua bidest. After incubation during 10 min. at 37°C, tubes were chilled to 0°C, 1.0 ml 1 N Na_2CO_3 was added and the absorbance at 410 nm was measured. Blanks received 1.0 ml 1 N Na_2CO_3 before the incubation at 37°C and after chilling at 0°C 0.2 ml substrate solution were added.

F. The determination of DNA, RNA and protein

1. In crude enzyme extracts

Protein content was determined in the crude enzyme extract after precipitating the proteins with 5% trichloroacetic acid and dissolving the pellet in 1 N NaOH for the determination of protein according to Lowry et al. (1951) adapted to the high alkaline conditions.

2. In liquid cultures of microplasmodia

DNA, RNA and protein were determined in 4 ml aliquots from the suspension cultures. Pre-extraction of low-molecular weight material was carried out as described by Sachsenmaier and Rusch (1964). The washed pellets were hydrolysed in 0.5 N $HClO_4$ at 70°C during 30 min. and centrifuged during 5 min. at 1200 g. The hot $HClO_4$-insoluble pellet was washed once with 0.5 N $HClO_4$ and the two supernatants were combined. DNA content was determined in the 0.5 N $HClO_4$-extract according to Burton (1956). RNA was calculated from the difference of the UV absorption at 268.5 nm - 320 nm after the proportion originating from DNA had been deducted from the total UV absorption (Pieck et al., 1971). This method was more convenient and gave more reproducible results than the orcinol method. With the orcinol method carried out as described by Sachsenmaier and Rusch (1964) we obtained higher RNA contents probably due to non-nucleic acid compounds

which gave a reaction with orcinol (Wanka, 1962). The acid-insoluble residue was dissolved in 1 N NaOH and used for the protein measurements according to Lowry et al. (1951) adapted to the high alkaline conditions.

3. In nuclear preparations

To measure DNA, RNA and protein contents nuclear pellets were washed once with 0.25 N $HClO_4$, once with 8% CCl_3COOH in acetone and twice with 0.25 N $HClO_4$. Determination of DNA, RNA and protein was carried out as described under F.2.

G. The isolation of nuclei

Two isolation media were used to obtain nuclei:

1. sucrose medium according to Mohberg and Rusch (1971): 0.25 M sucrose, 10 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.1 and 0.1% (v/v) Triton X-100.
2. salt medium: 30 mM NaCl, 5 mM $MgCl_2$, 1 mM KCl, 10 mM Tris/HCl, pH 7.1 and 0.1% (v/v) Triton X-100. These concentrations of salts were obtained after squashing pieces of macroplasmodia with various concentrations of salts and mixtures of salts. Concentrations and mixtures were varied until nuclei had the same appearance under the phasecontrast microscope as the nuclei that can be seen in smear preparations according to Guttes et al. (1961) (Derksen, unpublished results).

Nuclear isolation was carried out essentially according to Mohberg and Rusch (1971) changed in minor details. Microplasmodia were collected by centrifugation (2 min. at 500 g) and washed twice briefly with ice-cold aqua bidest. Microplasmodia from one culture were suspended in 200 ml nuclear isolation medium and homogenized during 30 seconds in a Waring Blendor set at 100 V. After 15 min. at 0°C for the foam to settle the homogenate was centrifuged at 90 g during 5 min. and the 90 g supernatant was filtered over two layers of milk filter (Milac, Brocades N.V., Nijmegen) wetted in nuclear isolation medium. The filtrate was centrifuged for 10 min. at 1200 g and the nuclear pellet was washed 3 times by suspending the nuclei in 20 ml nuclear isolation medium with a 20 ml syringe and a needle with 1 mm diameter, and pelletting the nuclei with centrifugation at 1200 g during 5 min. Recovery of nuclei can be

increased by again homogenizing the 90 g pellet and combining the first and the second 90 g supernatants. Nuclei were counted in a hemocytometer with a phasecontrast microscope (Wild) set at 480 x magnification. Material to be examined with the electron microscope was fixed at room temperature in 2% glutaraldehyde in 0.05 M sodium cacodylate/HCl, pH 7.1 and postfixed with OsO_4 according to Palade (1952).

CHAPTER III

A pH - DEPENDENT NUCLEOLYTIC ACTIVITY IN COMMERCIAL SAMPLES OF DNA - A WARNING

Introduction

Many authors use commercial samples of DNA as substrate for the determination of the DNase activity of their enzyme preparations. And several authors used salmon sperm DNA (e.g. Jorgensen et al., 1966; De Petrocellis and Parisi, 1972; Kasunuma, 1973; Suhara, 1973). We also (this thesis) used salmon sperm DNA to identify the several DNases in the slime mold Physarum polycephalum.

During our study we found in our samples of salmon sperm DNA a DNA-degrading activity with a sharp maximum at pH 9.0, to which we found no reference in the literature. We studied this in other commercial samples of DNA to see if this activity was a general phenomenon.

Materials and methods

Salmon sperm DNA was obtained from Sigma (Lots no. 47B-7400, 47B-7740, 470-7400), from Schwarz/Mann (Lot no. X-1851), from Carl Roth (Lot no. 220215) and from Calbiochem (Lot no. 010184). Calf thymus DNA, type I (Lot no. 82C-9500) and type V (Lot no. 99B-0080 and 113C-1790) and Bovine serum albumin, type V-powder (Lot no. 112C-2410) were all obtained from Sigma.

DNA was swollen and dissolved in sterile aqua bidest. overnight at 4°C before the incubation. Denaturation of the DNA was achieved by heating the DNA solution at 95 - 100°C during 15 min. and than quickly cooling to 0°C in melting ice. Normally all solutions were made freshly before every incubation.

The incubation mixture to measure degradation of the DNA consisted of: 1.0 ml 0.3 M buffer, 0.2 ml DNA (5 mg/ml) and 0.2 ml $MgCl_2$-solution, or aqua bidest. Mostly the mixture contained 1.0 ml 0.3 M glycine/NaOH-buffer, pH 9.0 and 5 or 7.5 mM $MgCl_2$.

Incubation was carried out at 30°C during 90 min. After the incubation the test tubes were chilled in ice and 0.1 ml bovine serum albumin (25 mg/ml aqua bidest.) and 1.0 ml 2.5 N $HClO_4$ were added. The tubes were shaken vigorously and after 10 - 15 minutes at 0°C they were centrifuged at 1500 g during 10 min. The extinctions of the

supernatants were measured at 260 nm and were corrected for blanks which were obtained by adding the DNA to an incubation mixture incubated at 30°C and chilled to 0°C before adding albumin and $HClO_4$.

Degrading activities were expressed as percentage of the substrate hydrolyzed per minute. The molar absorption coefficient of deoxyribonucleotides was assumed to be 10200 (Beaven et al., 1955) and the average molecular weight 340 (Baddiley, 1955).

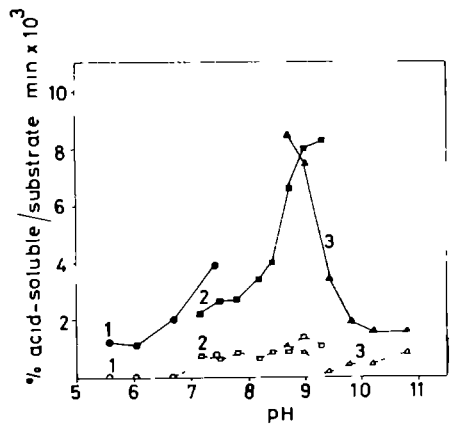


Fig. 6

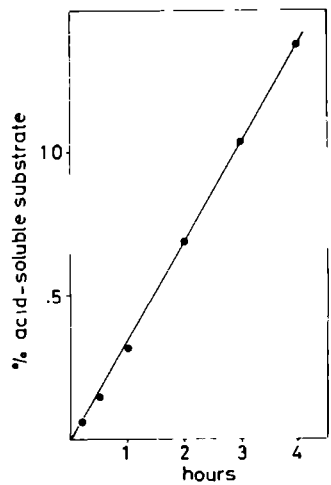


Fig. 7

Figure 6. Effect of pH on the degradation of salmon sperm DNA (type III, Sigma).
Degrading activity was tested in 5 mM $MgCl_2$. The following buffers were used: 1 Na-cacodylate/HCl, 2 Tris/HCl, 3 glycine/NaOH.
●—●, ■—■, ▲—▲,: native DNA; O—O, □—□, △—△,: denaturated DNA.

Figure 7. Formation of acid-soluble products of salmon sperm DNA (Sigma) as a function of the incubation time.
Degrading activity was measured in glycine/NaOH, pH 9.0 + 5 mM $MgCl_2$.

Results

Figure 6 shows that in the alkaline region native salmon sperm DNA of Sigma is degraded to acid-soluble products. Maximum degradation

occurs near pH 9.0. Denaturated DNA showed very little degradation, moreover without a clear pH-optimum under the same conditions.

In the acid pH region, namely in Na-formate/formic acid buffer, pH 2.5 - 4.2 and Na-acetate/acetic acid buffer, pH 3.8 - 5.6, we found no hydrolyzing activity in native or denaturated salmon sperm DNA.

Although we found some degrading activity in the other DNA samples, these activities possessed no optimum activity in the alkaline pH region, they were lower and less reproducible (table III).

Table III. Degrading activity in several commercial native DNA species in glycine/NaOH, pH 9.0 and 5 mM $MgCl_2$.

DNA	Manufacture	% substrate hydrolysed/min. x 10^3
salmon sperm	Sigma	5.79 ± .98 (7)
salmon sperm	Schwarz/Mann	2.71 ± .77 (3)
salmon sperm	Carl Roth	1.85 ± .49 (3)
salmon sperm	Calbiochem	1.13 ± .42 (2)
calf thymus,I	Sigma	1.25 ±1.15 (3)
calf thymus,V	Sigma	4.35 ±1.67 (3)

average ± standard deviation (number of measurements)

As figure 7 shows, the degradation of the native salmon sperm DNA of Sigma was linear with the incubation time. Sterilization of all solutions did not affect the degrading activity, so DNA degradation due to microbial contamination was very unlikely. The linear time course points in the same direction.

As figure 8 shows, maximum degradation of native salmon sperm DNA of Sigma occurs at about 5 - 10 mM $MgCl_2$. Higher Mg^{2+}-concentrations were inhibitory.

We obtained highest degradation of native salmon sperm DNA of Sigma with Mg^{2+}-cations in the incubation mixture, but Zn^{2+}, Cu^{2+} and Mn^{2+} also stimulated the DNA degradation; Ca^{2+} was strongly inhibitory and EDTA had hardly any effect (table IV). The different effects of these cations and of EDTA on the degradation of calf thymus DNA, type I and V suggest that these degrading activities are different from that in salmon sperm DNA (table IV).

Table IV. Effect of various divalent cations and of EDTA on the degrading activity in three native DNA species (Sigma). The concentrations of all cations and of EDTA was 7.5 mM. Hydrolytic activities are expressed as percentages of the native substrate hydrolysed without extra ions.

ion	salmon sperm	calf thymus, type I	calf thymus, type V
none	100%	100%	100%
$MgCl_2$	510	278	51
$ZnCl_2$	176	0	86
$CuCl_2$	163	0	105
$CaCl_2$	0	89	42
$MnCl_2$	276	86	49
EDTA	110	0	172

With incubation at 0°C there is hardly any degradation of salmon sperm and calf thymus, type I DNA, but the degradation of calf thymus, type V DNA (table V) is the same as at 30°C. This gives more evidence that the degrading activities in salmon sperm DNA and calf thymus, type V, are different.

With further purification by means of alcohol precipitation, phenol extraction, or dialysis of the salmon sperm DNA of Sigma we could only partially remove the degrading activity from the DNA (table VI). The fact that the degrading activity is removed partially with dialysis and not completely with phenol extraction suggests a non-protein character of the degrading principle.

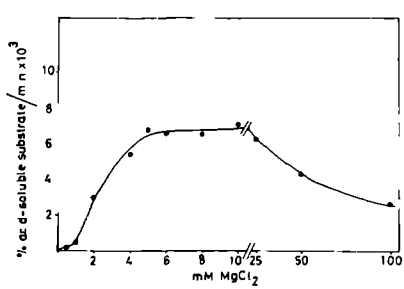


Figure 8. Effect of the $MgCl_2$-concentration on the degradation of salmon sperm DNA (Sigma). Degrading activity was measured in glycine/NaOH, pH 9.0

Table V. Formation of acid-soluble products at 0°C from three native Sigma DNA species.
Degrading activities were tested in glycine/NaOH, pH 9.0 + 7.5 mM $MgCl_2$. Degrading activities are expressed as percentage of the activities at 30°C.

DNA	% of degrading activity at 30°C
salmon sperm	14
calf thymus, type I	19
calf thymus, type V	88

Table VI. Effect of three purification methods on the degrading activity in native salmon sperm DNA (Sigma).
Degrading activity was tested in glycine/NaOH, pH 9.0 + 7.5 mM $MgCl_2$. The activities are expressed as percentage of the activity of the unpurified salmon sperm DNA. Dialysis was carried out against a 100 x greater volume aqua bidest. + 1 ml chloroform at 0°C, the water was changed twice. Phenol extraction was carried out according to Kirby (1956) after dissolving the DNA in 0.03 M Tris/HCl, pH 8.0 + 1.0% Na-dodecylsulfate.

Purification method	% of degrading activity in unpurified DNA
dialysis against aqua bidest.	40
precipitation with ethanol	64
phenol extraction	20

Discussion

Taken together our experiments demonstrate the existence of a degrading activity in native salmon sperm DNA manufactured by Sigma, which activity is strongly stimulated by Mg^{2+}-ions and with maximum activity at pH 9.0.

In heat-denaturated samples no activity could be demonstrated. This means that either the degrading principle is not active on denaturated DNA, or that this principle is a protein, or at least heat-labile.

However, the degrading entity is probably not a protein. The presence of salmon testis deoxyribonuclease in the salmon sperm DNA would be very unlikely because of the low stability of this enzyme especially at pH 8.5 and 30°C (Yamamoto, 1972).

In other commercial samples of DNA nucleolytic activities - not studied very extensively yet - were demonstrated as well. They possessed, however, no clear pH optimum in the alkaline range as was observed for the Sigma salmon sperm DNA.

All these activities can interfere with measurements of activities of deoxyribonuclease, especially if enzyme solutions of low activities are studied.

CHAPTER IV

ACTIVITIES OF DNA DEGRADING ENZYMES IN THE SLIME MOLD PHYSARUM POLYCEPHALUM. EVIDENCE FOR FIVE DIFFERENT ENZYMES

Introduction

In many organisms different types of DNases are found. Mostly they differ in requirements for mono- and divalent cations and have their maximum activity at different pH's (see reviews by Laskowski, 1967 and Lesca, 1971). In mammalian tissues for instance DNase I has its maximum activity at pH 7.1 and has an absolute requirement for divalent cations (Lindberg, 1967) and DNase II with its maximum activity at pH 5, needs no divalent cations (Bernardi, 1968). We wondered therefore if DNases could be found in Physarum polycephalum with their maximum activities at different pH's and need of different cations.

Hiramaru et al. (1969a) mentioned the existence in crude enzyme extracts of Physarum of a DNase having its maximum activity at pH 4.5. But these authors described no details and didn't present data showing the relation between pH and DNase activity in crude enzyme extracts. In this chapter we present indications for the existence in crude enzyme extracts of five DNA degrading activities working at maximum rate at different pH's.

Results

Figure 9 shows the presence in the crude enzyme extract of five peaks in DNase activity dependent on the pH of the incubation mixture, namely at pH 3.2 with a preference for native DNA and around pH 3.4, 4.0, 7.6 and 8.5, with a preference for denaturated DNA. The DNase activities per mg protein at the acid pH's were 10 - 20 times higher than those at the alkaline pH's. Omitting $MgCl_2$ from the incubation mixture at the alkaline pH's did not affect the pH value where maxima in DNase activity were observed, only the activity was lower.

DNase activity was strongly influenced by the buffer used in the incubation mixture. DNase activities measured in sodium formate were greater than in sodium acetate. Incubation mixtures containing sodium cacodylate exhibit greater DNA degradation than mixtures containing Tris/HCl, or glycine/NaOH buffers (figure 9). 0.2 M Sodium phosphate, 0.3 M PIPES and 0.3 M Tris/maleate buffers were strongly inhibitory

Table VII. Effect of various divalent cations and of EDTA on deoxyribonuclease activity.
The incubation mixture received 0.1 ml of an appropriate divalent cation solution to reach the desired final concentration (1, 5, or 10 mM). Controls received 0.1 ml aqua bidest. Activities are expressed as percentage of the activity without additions. Nat.DNA: native DNA, den.DNA: denaturated DNA.

ion	final concentration (mM)	activity in % of the control				
		pH 3.2 nat.DNA	pH 3.2 den.DNA	pH 4.0 den.DNA	pH 7.6 den.DNA	pH 8.5 den.DNA
Mg^{2+}	1	0	90	95	116	124
	5	0	80	94	136	104
	10	0	58	68	122	82
Mn^{2+}	1	0	0	82	121	171
	5	0	0	60	114	186
	10	0	0	52	96	158
Ca^{2+}	5	0	0	74	38	31
	10	0	0	70	21	18
Cu^{2+}	5	0	7	5	16	63
	10	0	2	0.5	26	61
Zn^{2+}	1	200	209	80	31	86
	5	184	209	92	14	8
Co^{2+}	5	11	73	58	0	0
	10	23	20	35	0	0
EDTA	5	71	60	27	0	0
	10	61	81	26	0	0

The alkaline DNase activities are stimulated by the administration of Mg^{2+} and Mn^{2+} ions, although in different ways (table VII). The administration of other divalent cations proved to be very inhibitory. Administration of EDTA shows that the alkaline DNase activities have an absolute need for divalent cations.

Divalent cations inhibit the acid DNase activities (table VII, except Zn^{2+} which has a great stimulatory effect on the DNase activities at pH 3.2 (table VII). Inhibition of the acid DNase activities after administration of EDTA shows that these activities also need divalent

(2–20% of the activity measured in 0.3 M Tris/HCl buffer).

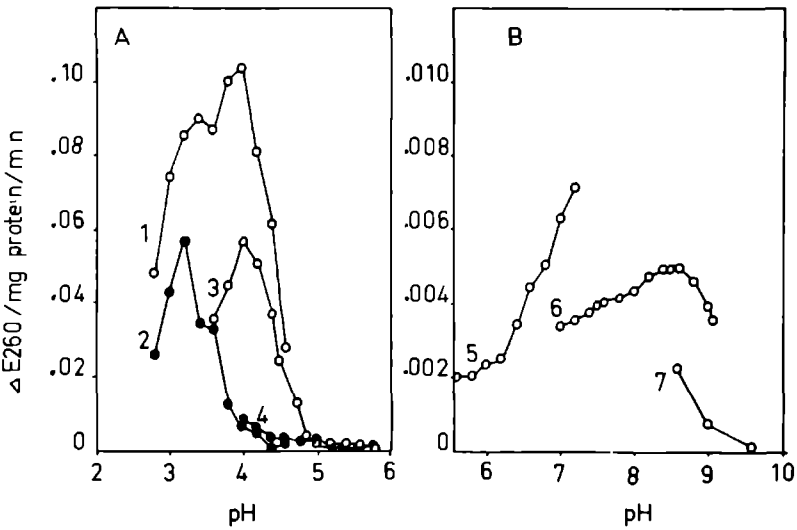


Figure 9. A and B. Effects of substrate, buffer and pH on deoxyribonuclease activity.
All buffers are 0.3 M. The alkaline incubation mixtures (B) contained 5 mM $MgCl_2$. The following buffers and substrates were used: 1. Sodium formate/formic acid + denaturated DNA, 2. Sodium formate/formic acid + native DNA, 3. Sodium acetate/acetic acid + denaturated DNA, 4. Sodium acetate/acetic acid + native DNA, 5. Sodium cacodylate/HCl + denaturated DNA, 6. Tris/HCl + denaturated DNA, 7. glycine/NaOH + denaturated DNA.

At alkaline pH's the crude enzyme extract had a preference for denaturated DNA. The degrading activities on native DNA could not be demonstrated in a reproducible way, the amount of acid soluble products did not increase linearly with the amount of enzyme, nor with the incubation time. Possibly the observed increase of acid soluble products after digestion of native DNA is the result of degradation of single stranded pieces in the commercially obtained salmon sperm DNA. The DNase activities on native DNA at pH 3.2 and on denaturated DNA at pH 3.4, 4.0, 7.6 and 8.5 were linearly proportional to the enzyme concentration and the incubation time under the conditions used.

cations, although the increase of the pH 3.2 DNase activity on denaturated DNA after administration of 10 mM EDTA with regard to the administration of 5 mM EDTA suggests that this enzyme is inhibited by some divalent cations present in the crude enzyme extract.

Figure 10 shows that the DNase activities become inactivated at higher temperatures but in different ways. Especially the pH 3.2 activity on denaturated DNA proved to be very labile.

DNase activity at pH 3.2 on native DNA is more stable towards heat inactivation (figure 10) and during the incubation conditions (figure 11) than the DNase activity on denaturated DNA. The DNase activities on denaturated DNA at pH 4.0, 7.6 and 8.5 are stable during the incubation conditions.

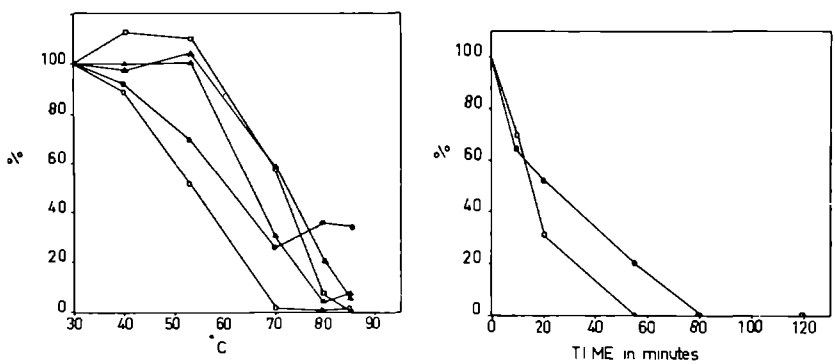


Figure 10. Effect of heat treatment on the DNA degrading activities. Portions of a crude enzyme extract at pH 7.1 were heated during 10 min. at different temperatures. After chilling the extracts to 0°C the DNase activities were measured as mentioned under chapter II.
The symbols indicate: —●—: pH 3.2 + denaturated DNA, —○—: pH 3.2 + native DNA, —□—: pH 4.0 + denaturated DNA, —△—: pH 7.6 + denaturated DNA, —▲—: pH 8.5 + denaturated DNA.

Figure 11. The stability of the deoxyribonuclease activities at pH 3.2 under the incubation conditions.
Pre-incubation was carried out as follows: 1.0 ml 0.3 M sodium formate/formic acid, pH 3.2 + 0.2 ml enzyme extract was put at 30°C for different periods. After the pre-incubation time indicated, incubation was started immediately by adding 0.2 ml DNA solution (5 mg/ml aqua bidest.) to the incubation mixtures and shaking the test tubes. Incubation was carried out during 10 min. at 30°C. Blanks received DNA after the incubation and chilling of the test tubes. The symbols indicate:
—●—: native DNA, —○—: denaturated DNA.

Discussion

Three arguments are presented here to suggest the existence of at least two different alkaline DNA degrading nucleases.

1. The occurrence of two optima of enzyme activity in Tris/HCl buffer (figure 9, curve 6).
2. The pH 7.6 and 8.5 activities are stimulated to different degrees by Mg^{2+} and Mn^{2+} ions (table VII).
3. The two activities have different stabilities towards higher temperatures (figure 10).

Perhaps the inhibitory effect of sodium phosphate on the alkaline DNase activities accounts for the observation of Hiramaru et al. (1969a), who used potassium phosphate during the isolation of their crude enzyme extracts, of a DNase activity on denaturated DNA only at pH 4.5 in their enzyme extracts.

Evidence for the existence of two different acid DNase activities, which degrade denaturated DNA comes from the following observations.

1. Zn^{2+} stimulates the activity at pH 3.2 and inhibits the activity at pH 4.0 (table I).
2. The pH 3.2 activity is more labile at higher temperatures than the pH 4.0 activity (figure 10).

Around pH 3 native DNA becomes partly denaturated depending on the DNA concentration, ionic strength and the temperature of the DNA solution (Cavalieri et al., 1956; Dove et al., 1959; Bunville, 1960). In our incubation mixture salmon sperm DNA starts to denaturate around pH 3.4 and denaturation is complete around pH 2.8 as judged by the increase of absorbance at 260 nm as a function of the decreasing pH of the incubation mixture. So it cannot be ruled out that the native DNA degrading enzyme needs partially denaturated DNA as substrate. And the pH optimum at pH 3.2 could be achieved by an increasing amount of partially denaturated DNA substrate and a decreasing enzyme activity as a function of lowering the pH of the incubation mixture. As figure 11 shows the enzyme is labile under the incubation conditions. However, the different stabilities towards heat treatment (figure 10) and incubation conditions (figure 11) suggest the existence of two different DNA degrading nucleases working at pH 3.2, one with a preference for native, or partially native and one for denaturated DNA.

The DNase activities at alkaline pH's are different from the activities at acid pH's in the following respects: Mg^{2+} and Mn^{2+} ions

stimulate the alkaline and inhibit the acid activities (table VII); Zn^{2+}, Co^{2+} and EDTA have greater inhibitory effects on the alkaline than on the acid activities (table VII); the inhibitory effect of Ca^{2+} on the acid activities is greater than on the alkaline activities (table VII); furthermore, the different nucleases exhibit different heat inactivation curves (figure 10).

All data therefore point to the existence in crude extracts of at least five different enzymes able to degrade DNA.

CHAPTER V

DNA DEGRADING ENZYMES OF THE SLIME MOLD PHYSARUM POLYCEPHALUM. PARTIAL CHARACTERIZATION OF THE ENZYMES IN CRUDE ENZYME EXTRACTS

Introduction

As described in chapter IV at least five DNA degrading enzymes exist in the slime mold Physarum polycephalum: one activity at pH 3.2 active on native DNA, and four others degrading denaturated DNA at about pH 3.4, 4.0, 7.6 and 8.5.

However, still uncertain is which of these activities belongs to aspecific nucleases, or is effected by other enzymes which are related to these enzymes, e.g. phosphodiesterase or phosphatase.

We therefore studied the RNA degrading activities, phosphodiesterase and phosphatase activities in relation to the pH of the incubation mixture, as it was done for the DNA degrading activities.

To obtain more certainty about the different identity of the activities cell fractionation studies were undertaken and the enzymes were separated by polyacrylamide gel electrophoresis.

Materials and methods

1. The materials and methods not mentioned below are described in chapter II.
2. Cell fractionation was carried out as follows. Microplasmodia were washed twice with aqua bidest. and once with fractionation medium (FM): 0.25 M sucrose, 10 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris/HCl, pH 7.1. Microplasmodia were homogenized with 5 up and down strokes in a teflon glass Potter-Elvehjem type homogenizer equipped with a Tri R - Stir R. model S 63 C - motor set at stage 3.5. The homogenate was centrifuged for 5 minutes at 90 g and the pellet was removed. The 90 g supernatant was centrifuged for 10 minutes at 2000 rpm in a Sorvall SS-34 rotor ($\pm$ 500 g). The pellet was washed once by resuspending it in FM and recentrifuging the suspension: fraction B. The 500 g supernatant was centrifuged during 10 minutes at 5000 rpm ($\pm$ 2500 g). The pellet was washed once: fraction M. The 2500 g supernatant was centrifuged during 10 minutes at 13.000 rpm ($\pm$ 20.000 g). The pellet was washed once: fraction L. The 20.000 g supernatant was centrifuged during 60 minutes at 40.000 rpm in a

Spinco 50 rotor (100.000 g). The pellet is called fraction P and the supernatant fraction S.

The fractions and total microplasmodia were suspended in homogenization medium and crude enzyme extracts were prepared as described under chapter II. Fraction S was brought to 0.1% (v/v) Triton X-100 and treated further as the other fractions.

Fraction N consisted mainly of nuclei according to Mohberg and Rusch (1971) and Grant and Poulter (1973). Fraction M consisted mainly of mitochondria according to Brewer (1967) and Grant and Poulter (1973). Fraction L was not identified, but probably consists of light mitochondria, food vacuoles, dictyosomes and perhaps lysosomes. Fraction P consisted mainly of ribosomes and polyribosomes according to Mittermayer et al. (1966), Zellweger and Braun (1971) and Brewer (1972). Fraction S was called the cytosol.

3. To separate the DNA and RNA degrading enzymes two electrophoretic systems were used, both based upon the polyacrylamide electrophoresis system of Davis (1964).

A. DNA degrading enzymes.

Electrophoresis was carried out according to Boyd and Mitchell (1965) with the following small modifications: 1. the gels contained 0.75 mg calf thymus DNA, type I (Sigma) per ml, instead of 1 mg per ml, 2. to solution B 0.46 ml TEMED was added, 3. electrophoresis was performed during 70 minutes, 4. preincubation lasted 45 minutes, instead of 30 minutes; the incubation buffers were 0.1 M (same buffers as were used for the determination of the DNA degrading activities); during preincubation buffers were changed once; incubation lasted 60 minutes, 5. during pre-elution ("gel soaking") the buffer was changed twice, instead of once; during elution the buffer was changed once after 15 hours.

Crude enzyme extracts were centrifuged during 60 minutes at 100.000 g and, before electrophoresis, diluted 1 : 1 with 30% (w/w) sucrose. 100 µl extract-sucrose mixture was put on the gel. Gels were scanned on a Kipp Densitometer DD2 supplied with a Kipp recorder BD 8 with filter D (transmission range 580 - 650 nm), set at transmission.

B. RNA degrading enzymes.

Electrophoresis was carried out according to Davis (1964). Incu-

bation and detection of the RNase activities were performed according to Wilson (1969). Crude enzyme extracts were brought to 5% (w/v) sucrose and 100 µl of this sucrose homogenate were put on a polyacrylamide gel. Incubation was carried out for 30 minutes at 30°C in 0.6 M buffer containing 4 mg yeast RNA/ml (Schwarz).

4. The acid soluble products after degradation of salmon sperm DNA with crude enzyme extracts were separated with DEAE-cellulose chromatography (Diethylaminoethyl Cellulose, DE 32 standard, Whatman) according to Tomlinson and Tener (1963). Incubation mixtures consisted of 15 ml crude enzyme extract, 5 ml buffer (3.5 M), 4 ml $MgCl_2$ solution (final $MgCl_2$ concentration was 1 mM for the pH 8.5 activity and 5 mM for the pH 7.6 activity) or aqua bidest., 35 ml salmon sperm DNA (10 mg/ml aqua bidest.). After incubation at 30°C during 20 minutes for the acid activities, or 30 minutes for the alkaline activities, 7 ml of 60% $HClO_4$ were added. After 15 minutes at 0°C the solution was clarified by centrifugation during 10 minutes at 1000 g. The supernatant was brought to pH 7.0 with 50% KOH and after 15 minutes at 0°C the solution was clarified by centrifugation during 10 minutes at 1000 g. The supernatant was brought to pH 4.7 with sodium acetate (2.5 M) and put on the column. After pre-elution with 2.5 mM sodium acetate buffer (pH 4.7) plus 7 M urea, the column was eluted with 2.5 mM sodium acetate buffer (pH 4.7) plus 7 M urea containing a linear gradient of 0 to 0.5 M NaCl (total volume 1000 ml).

Two enzymes were taken as reference: beef pancreas DNase I, EC 3.1.4.5. (Sigma, type DN-100) and endonuclease from Neurospora crassa, EC 3.1.4.- (Boehringer). The incubation mixture for DNase I consisted of 2 ml DNase I (7.5 mg/ml aqua bidest.), 5 ml buffer (0.17 M sodium acetate, pH 5.0 plus 16 mM $MgCl_2$) and 7.5 ml denaturated salmon sperm DNA (10 mg/ml aqua bidest.). Incubation was carried out during 25 minutes at 25°C. Endonuclease was incubated during 30 minutes at 37°C; the incubation mixture consisted of 0.25 ml endonuclease (1 mg/ml aqua bidest.) plus 20 ml denaturated salmon sperm DNA, 2 mg/ml 0.12 M Tris/HCl, pH 8.0 plus 12 mM $MgCl_2$ plus 0.2 M NaCl.

Results

1. Dependence on pH and buffer of the activity of RNase, phosphodiesterase and phosphatase activity in crude enzyme extracts

The RNA degrading activity possesses an optimum at pH 3.8 - 4.0 and no optimum or shoulder could be observed at pH 3.2 (figure 12). Although some activity exists in the alkaline region, no optimum was found. Figure 13 shows that phosphodiesterase activity shows maxima at pH 9 and at pH 4.0 - 5.0. Phosphatase has a maximal activity at pH 4 and a broad optimum was found at pH 9 - 10 (figure 14).

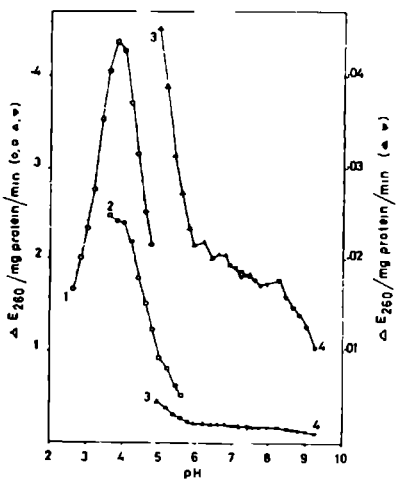


Fig. 12

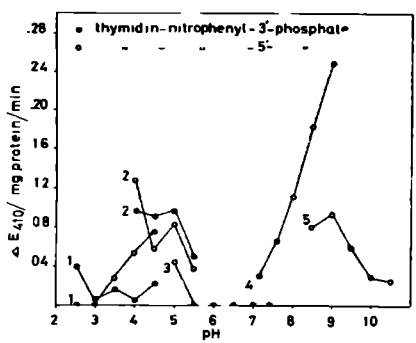


Fig. 13

Figure 12. Effect of buffer and pH on the RNA degrading activity in crude enzyme extracts.
All buffers were 0.3 M. 1 Sodium formate/formic acid, 2 sodium acetate/acetic acid, 3 sodium cacodylate/HCl, 4 Tris/HCl.

Figure 13. Effect of buffer and pH on the phosphodiesterase activity in crude enzyme extracts.
Buffer 5: glycine/NaOH.
Other buffers: see under figure 12.

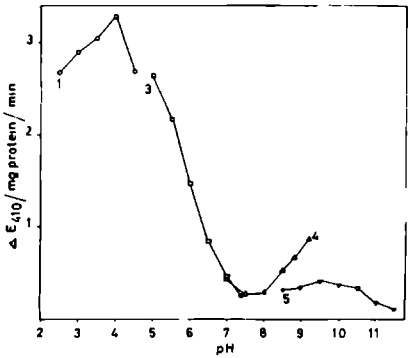


Figure 14. Effect of buffer and pH on the phosphatase activity in crude enzyme extracts.
For detail see figure 12.

2. Distribution of the DNA and RNA degrading activities over five cell fractions

From the effect of the pH on the DNA and RNA degrading activities we got evidence for the existence of at least one RNase, but several DNases. We tried to separate these activities by differential centrifugation of crude cell homogenates. Five fractions were prepared (see methods). Table VIII shows the activities of the DNA and RNA degrading enzymes in these fractions. The recoveries from the total microplasmodia were rather low, probably due to the removing of the 90 g pellet and the washing of the fractions N, M and L. Figure 15 presents the distribution of the relative specific activities in the fractions. Most of the activities were found in the fraction S.

In order to find an argument regarding the question whether one or different enzymes bring about the RNA and DNA degrading activities at different pH levels, the correlation coefficients between these activities over the fractions N, M, L and P were studied. E.g. the relative RNA degrading activities in the four particle fractions of two different cell extracts were calculated both for pH 3.2 and for pH 4.0. On the resulting 8 pairs of relative activities (one for pH 3.2 and one for pH 4.0) Pearson's correlation coefficient r was calculated. The same calculations were performed for other pairs of pH levels, both for RNase and DNase activities, and for both activities at the same pH level (table IX).

Table VIII. Distribution of the DNA and RNA degrading activities over five cell fractions.
Activity was expressed as percentage of the summarized activities of the fractions N + M + L + P + S.
Recovery was expressed as percentage of the total activity in the microplasmodia from which the cell fractionation was started.

enzyme	fraction N		fraction M		fraction L		fraction P		fraction S		recovery	
	exp.1	exp.2	exp.1	exp.2	exp.1	exp.2	exp.1	exp.2	exp.1	exp.2	exp.1	exp.2
DNase 3.2 - N	2.95	7.47	2.21	2.69	2.34	5.09	2.73	-	89.77	84.75	94.1	46.2
3.2 - D	2.89	5.38	2.51	2.55	1.34	.58	1.36	-	91.90	91.47	65.3	44.7
4.0	9.15	10.21	1.51	1.11	.90	.83	6.07	4.26	82.37	83.60	35.5	39.2
7.6	6.41	7.99	.82	0	0	1.26	20.49	22.03	72.28	68.72	22.1	20.7
8.5	6.95	8.12	2.63	3.42	2.93	5.44	18.84	17.75	68.63	65.27	44.1	39.6
RNase 3.2	2.20	3.58	.99	.89	.77	.55	2.77	2.58	93.26	92.40	107.2	90.8
4.0	4.26	5.88	1.73	1.61	1.71	1.37	4.47	3.67	87.84	87.46	44.0	48.8
7.6	7.93	8.77	2.93	3.01	2.04	1.38	8.64	6.69	78.46	80.14	24.6	31.5
8.5	4.42	6.67	2.58	2.17	1.94	.99	6.18	4.77	84.87	85.41	26.8	33.8
protein	13.48	14.53	4.17	2.64	2.14	1.57	13.25	9.81	66.96	71.45	45.0	31.1

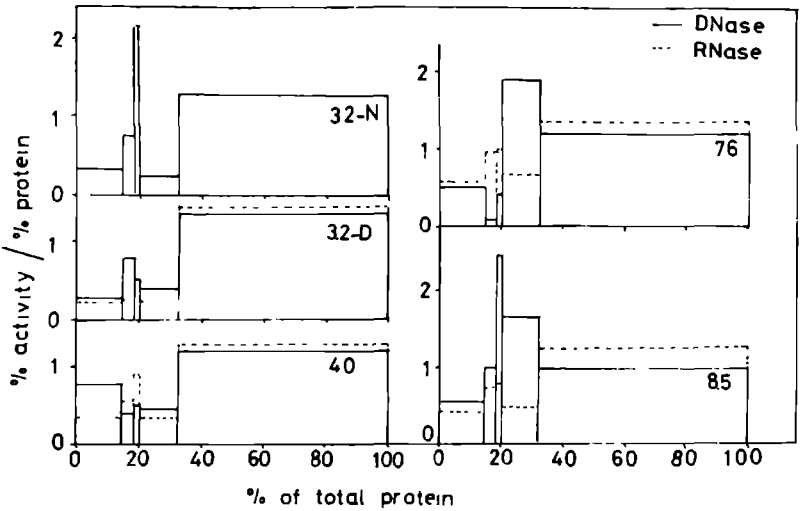


Figure 15. Distribution of relative specific activity of the DNA and RNA degrading activities over fractions N, M, L, P and S.

A high value of r (about .90 or over) was considered to be an indication for one enzyme responsible for both activities. A low value of r (about .70 or less) was considered to be a strong indication for different enzymes responsible for both activities, as such a low value of r does not significantly differ from 0 at the 5% level of significance. For r values between .70 and .90 it is somewhat dubious whether one should prefer the one or two enzyme assumption.

According to this criterion the values of r shown in table IX are in favour of one enzyme for the RNase activities at the different pH levels and of different enzymes for the DNase activities except for pH 8.5 and pH 7.6 and perhaps pH 4.0 and pH 3.2 - D. Comparing DNase and RNase activities a decision is more difficult; at the pH level 3.2-N vs. 3.2 two enzymes are more likely, at the level 4.0 one enzyme, but at the other levels there is only a slight preference for two enzymes.

Table IX. Correlation between the relative DNA and RNA degrading activities over the cell fractions N, M, L and P.

Between: RNase at different pH levels			Between: DNase at different pH levels			Between: DNase / RNase		
pH levels	r	n	pH levels	r	n	pH levels	r	n
4.0/3.2	.981	8	3.2-N/3.2-D	.573	7	3.2-N/3.2	.545	7
4.0/7.6	.866	8	4.0/3.2-D	.757	7	3.2-D/3.2	.716	7
4.0/8.5	.963	8	4.0/7.6	.450	8	4.0/4.0	.956	8
8.5/7.6	.970	8	4.0/8.5	.387	8	7.6/7.6	.724	8
			8.5/7.6	.988	8	8.5/8.5	.691	8

r = Pearson's correlation coefficient

n = number of pairs involved

3. Electrophoretic separation of the DNA and RNA degrading activities

To obtain more certainty about the different character of the DNA degrading activities we separated the proteins of the crude enzyme extract by polyacrylamide gel electrophoresis and the DNase activities in the gels were demonstrated according to Boyd and Mitchell (1965). Figure 16 shows a representative pattern of bands of one homogenate. The pattern of bands turned out to be very reproducible.

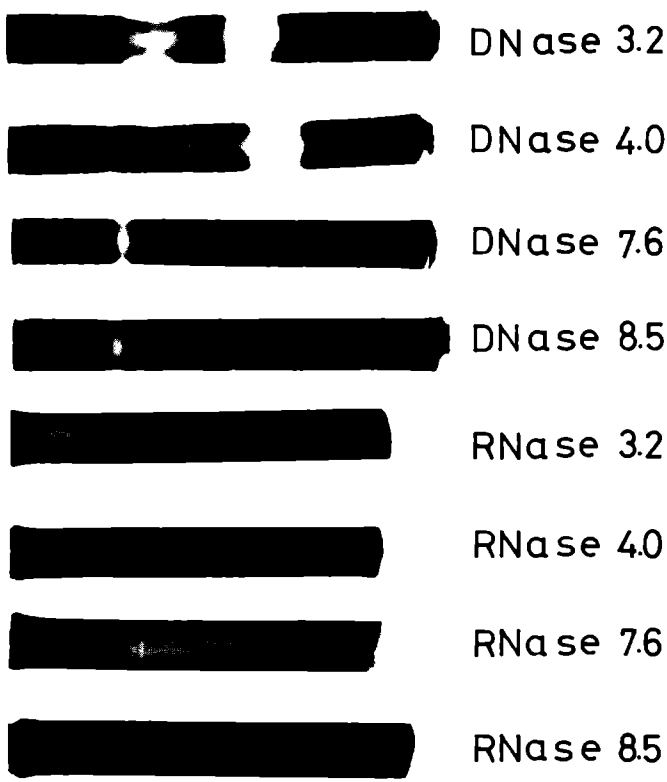
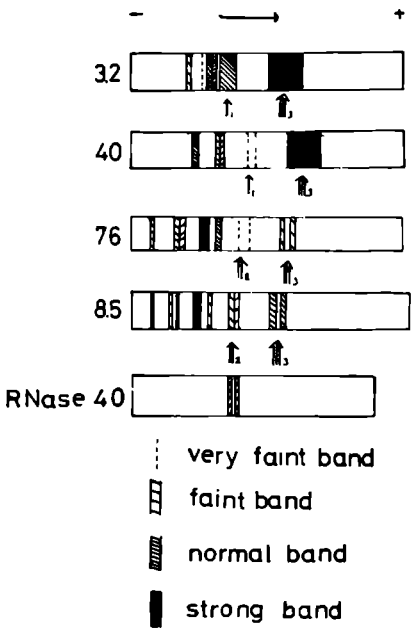


Figure 16. Electrophoretic separation of the DNA and RNA degrading activities.

The electrophoretic experiments were only performed with denaturated DNA enclosed in the gel. Experiments with native DNA in the gels, to detect the native DNA degrading activity at pH 3.2 were not completely successful until now, although some broad, vague bands are observed.

Figure 17 presents schematic drawings of the active zones in the gels. At pH 3.2 6 bands (1 very faint) and at pH 4.0 6 bands (2 very faint) are visible; at pH 7.6 9 bands (2 very faint) and at pH 8.5 9 bands are visible. The pH 3.2 activity possessed one broad band not present in the pH 4.0 activity (arrow 1). The pH 8.5 activity possessed 2 bands, which were very faint in the 7.5 activity (arrow 2). The acid activity possessed one strong and broad band (arrow 3), while the alkaline activities exhibited two bands in this position. The activities had many bands in common, but the relative intensities of these bands were different (see figure 18).



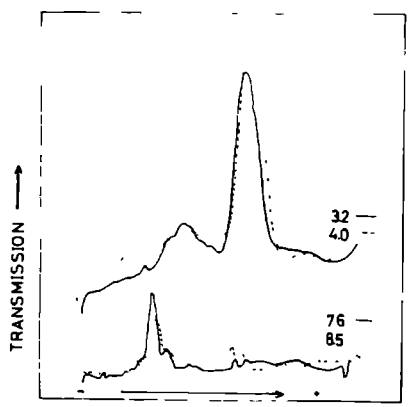


Fig. 17

Fig. 18

Figure 17. Schematic drawing of the pattern of bands obtained after electrophoretic separation of the DNA and RNA degrading activities.

Figure 18. Scanning of the gels containing DNase activity.

RNase activity could be detected in the gels in only two bands and this pattern did not change when the gels were incubated at different pH's, although in the alkaline region one of the two bands was very faint (see figure 16).

4. Mode of action of the DNA degrading enzymes

To know if the DNA degrading activities are exo- or endonucleolytic we separated the products of hydrolysis from salmon sperm DNA after incubation with crude enzyme extracts on a DEAE-cellulose column.

The results are presented in figure 19. The reaction products were compared to those of two other enzymes, namely pancrease DNase I which produces oligonucleotides and endonuclease from Neurospora crassa which produces mono- and oligonucleotides. Thus we were able to locate the mono- and oligonucleotides in the elution profile from the columns.

Figure 19 shows that all activities produces mixtures of mono- and oligonucleotides. However, differences do occur. At pH 3.2 more mononucleotides are produced from denaturated DNA compared to the production at pH 4.0. Profile 3 and 4 in figure 19 are two representative results of the 3.2 activities. The size of the mononucleotide peak varied from experiment to experiment at this pH. The pH 4.0, 7.6 and 8.5 profiles were far more reproducible.

Discussion

By extending the findings reported in chapter IV we collected more evidence for the existence of several DNases active in crude enzyme extracts of Physarum polycephalum.

The observed activities are interfered with by an aspecific nuclease with a maximum activity at pH 4.0 and phosphodiesterases with maximum activities at pH 9 and at pH 4.0 - 5.0.

The RNase activity decreases gradually from pH 4 onwards, while the DNase activity increases after pH 6 and decreases only after pH 8.6. So the DNA degrading activity at pH 4.0 could be from an aspecific nuclease, but at the other pH's DNases are active, or phosphodiesterases not active on RNA; however, part of the activities at pH 3.2, 7.6 and 8.5 could be achieved by the residual activity of aspecific nucleases having their maximal activity at pH 4.0. From the pH gradient, we expected the RNA degrading activity to be only one enzyme and this con-

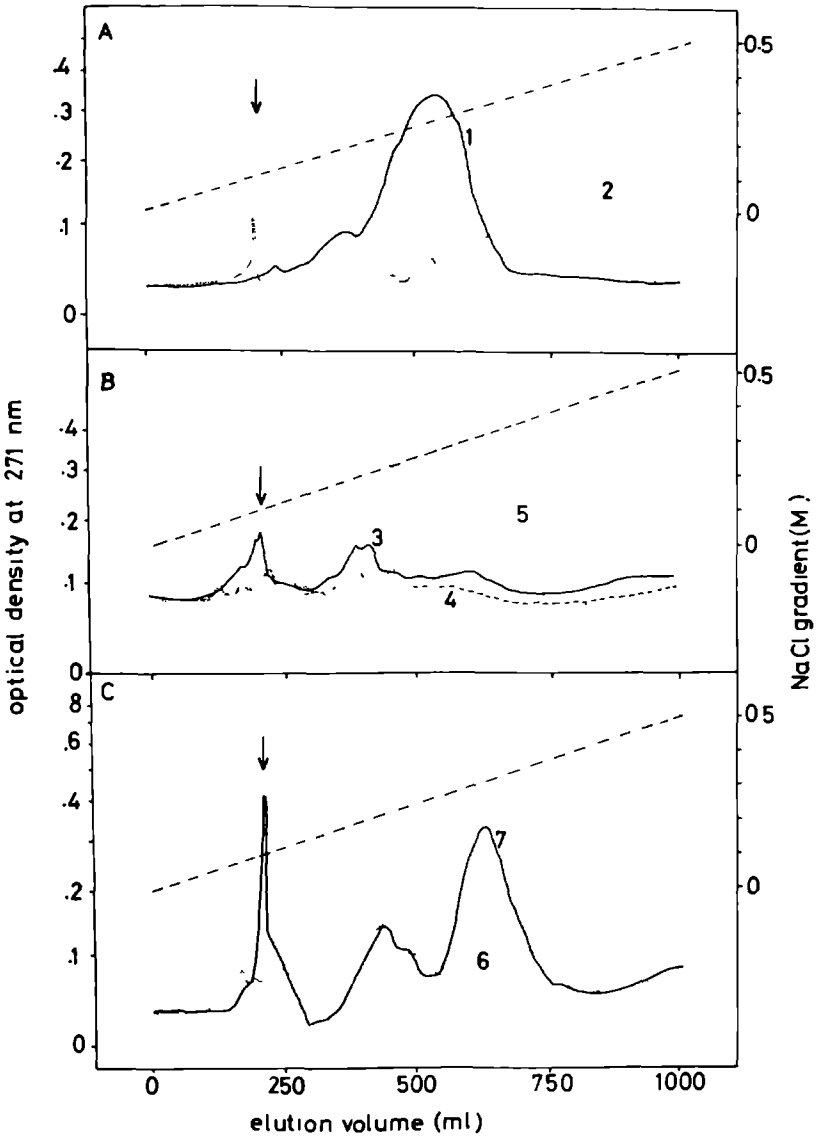


Figure 19. Chromatography on a DEAE-cellulose column of acid-soluble products from salmon sperm DNA formed during incubation at different pH's with crude enzyme extracts from Physarum or with beef pancreas deoxyribonuclease I and endonuclease from Neurospora crassa.
1. pancreas DNase I, 2. endonuclease from Neurospora crassa, 3. pH 3.2 activity on native DNA, 4. pH 3.2 activity on denaturated DNA, 5. pH 4.0 activity, 6. pH 7.6 activity, 7. pH 8.5 activity.
The arrow marks the position of the mononucleotides.

clusion was supported by the cell fractionation experiments.

Already Hiramaru et al. (1969) demonstrated the existence of two aspecific nucleases with maximum activity at pH 4.5, very near to pH 4.0 shown by our experiments. They homogenized their plasmodia in phosphate buffer and prepared their enzyme extracts by procedures completely different from ours (e.g. crude enzyme extracts were maintained overnight at 4°C in phosphate buffers). We found phosphate to be inhibitory at the alkaline activities and after standing at 0°C after 24 hours most of the DNA degrading activity was lost from the extracts. Perhaps this accounts for the differences in pH of the maximal activity between Hiramaru et al. (1969) and our findings.

The correlation coefficient between the relative activities of the DNase and RNase activities measured at the various pH's was calculated. Compared to the RNases the distribution of the DNases pos.ssed low correlation coefficients, except the correlation coefficients between the relative activities of DNase 7.6 and 8.5 and of DNase 4.0 and RNase 4.0.

A high correlation coefficient (> .9), however, does not mean per se that only one enzyme exists; two different enzymes can have the same association with the cell particles. But a low correlation coefficient (< .7) means that more than one enzyme is present. So evidence is obtained from the cell fractionation experiments that the DNA degrading activities at 3.2, 4.0 and in the alkaline region are achieved by different enzymes. That only one enzyme is responsible for the activities at pH 7.6 and pH 8.5 and for the RNase and DNase activities at pH 4.0 cannot be excluded from these experiments.

More evidence for the different identities of the DNA degrading enzymes was obtained from the electrophoretic experiments and these experiments showed very clearly that the observed DNA degrading activities are the added up activities of several different enzymes.

The chromatographic separation of the products of hydrolysis after incubation of DNA with Physarum crude enzyme extracts also shows the mixed character of DNA degrading activities at the several pH values.

The hydrolytic products of the pH 3.2 activities contained more mononucleotides than oligonucleotides as compared to the pH 4.0 activity. But the size of the mononucleotide peak of the 3.2 activities was not very reproducible; perhaps the products of these activities are affected by the strong phosphatase activity at this pH (see figure 14), which

splits off the phosphate group from the mononucleotides; the resulting mononucleosides do not adsorb onto the DEAE-cellulose column (Tomlinson and Tener, 1963). About half of the total UV absorbing material originally present was not adsorbed on the DEAE column. The final products at both pH's could be caused by an exonucleolytic activity having its maximal activity at pH 3.2 combined with an endonucleolytic activity having its maximal activity at pH 4.0.

The alkaline activities probably also are mixtures of endo- and exonucleases.

Conclusively one may say that the activities are brought about by mixtures of DNA degrading enzymes. Probably the mode of action of these enzymes can only be elucidated after purifying the different enzymes from the crude extracts.

With our electrophoretic system only two RNA degrading enzymes were detected and this pattern of bands did not change if the gels were incubated at different pH's. The enzymes had their maximum activity at pH 3.8 - 4.0 and they were distributed in the same way over the five cell fractions. However, more RNase isoenzymes exist in Physarum. Chet et al. (1973) demonstrated with the gel isoelectric focusing technique 12 RNase isoenzymes and Hiramaru et al. (1969) could isolate 4 specific RNases and 2 aspecific nucleases. But in crude enzyme extracts Hiramaru et al. (1969) found the RNA degrading activities to have one optimum activity only at pH 4.5.

Myxamoeba feed themselves by engulfing food particles and by digesting these particles within food vacuoles (Gray and Alexopoulos, 1968), and also in Physarum polycephalum pinocytosis was observed (Guttes and Guttes, 1960). Probably the slime mold needs this complex mixture of DNases and RNases to break down the various foreign nucleic acids which enter the cell by phagocytosis.

The cellular localization of the DNA and RNA degrading enzymes remains uncertain, because of the low recoveries of the enzyme activities (table VIII), probably due to losses of enzyme by removing of the 90 g pellet and the washing of the fractions N, M and L.

However, some preliminary conclusions can be drawn.

Although the highest activity of the nucleic acid degrading activities was found in the cytosol (perhaps partly due to leakage of the enzymes from the fractions N, M, L and P), compared to the RNA degrading activities the DNA degrading activities were more associated with the

cell particles. The pH 3.2 activity on native DNA seems to be partly localized in fraction L, probably containing light mitochondria, food vacuoles, dictyosomes and perhaps lysosomes according to the mitochondria isolation methods of Brewer (1967) and of Grant and Poulter (1973) and the polysome isolation methods of Brewer (1972), who named the 20.000 g - 10 min. fraction the post-mitochondrial fraction. Until now no lysosomes have been described in Physarum polycephalum.

The pH 7.6 activity seems to be partly localized in fraction P, i.e. the polysome fraction according to Zellweger and Braun (1971) and Brewer (1972). The pH 8.5 enzyme seems to be partly localized in fractions L and P. The pH 3.2 and 4.0 activities on denaturated DNA and the RNA degrading activities are mainly localized in the cytosol, fraction S. Probably the mixed character of the activities caused these complex distribution patterns.

In rat liver and many other tissues acid DNase is mainly associated with the lysosomes, and alkaline or neutral DNase belongs in rat liver to the mitochondria (De Duve et al., 1962). But in many other cell organelles from many organisms DNases have been characterized, e.g. from nuclei (Churchill, 1973; Hewisch, 1973; Slor, 1971), from chromatin (O'Conner, 1969), from mitochondria (Paoletti, 1972) and from chloroplasts (Egan and Carrell, 1972).

Until now no complete cell fractionation studies have been described for Physarum polycephalum, or other slime molds. Only the intracellular localization of acid phosphatase has been demonstrated by electron microscope cytochemistry. Kazama and Aldrich (1972) localized in Physarum flaviconum this enzyme in food vacuoles and dictyosomes and in Dictyostelium discoideum Gizelius (1971) localized acid phosphatase in the vacuoles and the cell surface.

Comparison of the cellular localization of the nucleic acid degrading enzymes as reported in this paper to other data is not possible.

Acknowledgements.

I wish to thank Drs. Ph. van Elteren for valuable statistical advices and Miss A.A.M. Rijken for purifying the yeast RNA.

ACTIVITIES OF DNA DEGRADING ENZYMES DURING GROWTH AND DIFFERENTIATION (SPHERULATION)

Introduction

In chapter IV and V we presented evidence for the existence of five different DNA degrading activities. Other organisms with multiple intracellular deoxyribonucleases show changes in enzyme levels under varying physiological conditions, which suggests that the different enzymes are involved with different processes in the cell (Shortman and Lehman, 1964). Many authors have related DNase activity to DNA synthesis or repair and to recombination processes in the cell (see reviews by Koerner, 1970 and Lesca, 1971).

The slime mold Physarum polycephalum is by its natural synchrony and by its capacity to undergo differentiation - spherulation or sporulation - a very useful tool for studying the relationship between various processes and enzyme activities (Hüttermann, 1973a). In this chapter we present the response of the DNA degrading activities in Physarum polycephalum to the different physiological conditions during the growth cycle, spherulation and during the mitotic cycle.

Results

1. DNA degrading activities in growing microplasmodia

The observation that the specific activities of the DNA degrading enzymes of freshly inoculated shake cultures were different from the ones of full-grown cultures prompted a more careful study of the variation in DNA degrading activities during the growth cycle.

Routinely 1 ml of a full-grown culture was added to 20 ml fresh medium and the stationary phase was obtained after about 72 hours (Werry, 1973). In the experiments described below we wanted to accelerate the entry into the stationary phase, therefore 3 ml of a full-grown microplasmodial culture were added to 20 ml fresh medium, and the stationary phase was obtained already after two days. The characteristics of these growth cycles are presented in figure 20. No lag-phase was observed; the growth was logarithmic until about 40 hours, with a doubling time of about 15 hours. Figure 21 shows that the DNA and RNA contents relative to the protein content fluc-

tuate during the growth cycle.

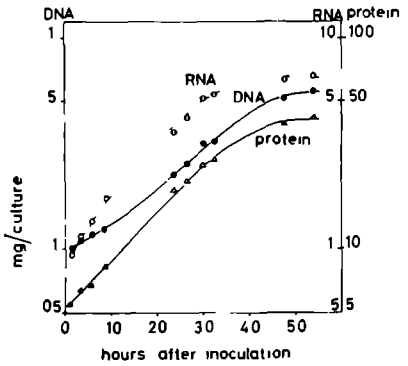


Fig. 20

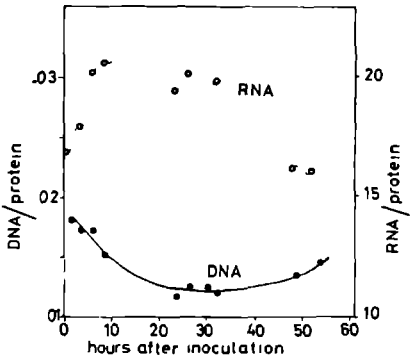


Fig. 21

Figure 20. DNA, RNA and protein content per 20 ml shaken liquid culture of growing microplasmodia in semi-defined medium.

Figure 21. DNA/protein and RNA/protein ratios during the growth cycle of microplasmodia.

The DNA/protein ratio decreased until about 25 hours and increased a little after that. The RNA/protein ratio increased until about 10 hours and decreased after about 25 hours.

Figure 22 presents the behaviour of the five DNA degrading activities during the growth of microplasmodia in shake cultures. The specific activities at acid pH's remain almost constant during the first part of the logarithmic phase, except the activity on denaturated DNA at pH 3.2 which decreases a little; during the second part of the logarithmic phase the acid activities increase 2-3 fold and after about 50 hours they decrease (figure 22A).

The alkaline specific activities (figure 22B) decrease sharply during the first 8 hours, remain low until about 25 hours and increase in the late logarithmic phase of the growth. As the stationary phase begins the specific activities level off.

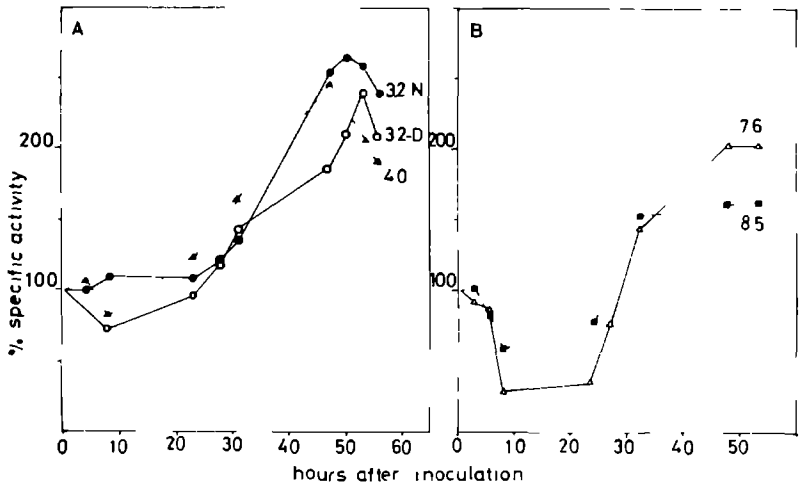


Figure 22. Specific activities of DNA degrading enzymes during the growth cycle of microplasmodia.
The data are expressed as percentage of the specific activity of growing microplasmodia at the time of inoculation (zero time).

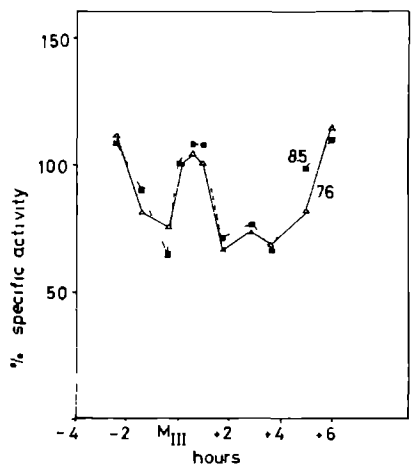


Figure 23. Specific activities of alkaline DNA degrading enzymes during the mitotic cycle. The data are expressed as percentage of the specific activity of the first harvest macroplasmodia after mitosis III (10-15 minutes after metaphase). This figure presents a typical experiment out of 6 similar experiments.

2. DNA degrading activities in growing macroplasmodia

Figure 23 shows the fluctuations in specific activity of the alkaline DNA degrading enzymes during the mitotic cycle of Physarum polycephalum. The activities at pH 7.6 and 8.5 exhibit three maxima in specific activity, namely during the early S-phase (0 - 1 hours after mitosis), the late S-phase (2 - 3 hours after mitosis) and in the mid G-phase (5 - 7 hours after mitosis). Very striking was the sharp increase in specific activity during the last part of the G-phase and the first part of the S-phase.

These maxima in specific activity were observed in all experiments, but the magnitude of decreases and increases of the activities varied from experiment to experiment. In big macroplasmodia (7 - 8 cm diameter) or plasmodia with late occurrence of mitosis III the changes in the specific activities were less pronounced than in small macroplasmodia (3 - 4 cm diameter).

Although the pH 3.2 activities also showed fluctuations in specific activity during the mitotic cycle, these fluctuations were not reproducible. The maxima in specific activity were found at the same time in the mitotic cycle as was observed for the alkaline activities, but these maxima were not observed in all experiments.

For the pH 4.0 activity only one reproducible maximum in activity was observed, namely in the mid G-phase (5 - 7 hours after mitosis) and the specific activity started to rise at 3 - 4 hours after mitosis. In some experiments a maximum at the end of the S-phase was found.

3. DNA degrading activities during sphérulation and germination of the spherules

Figure 24 and 25 present the changes in the DNA, RNA and protein content per shake culture of 20 ml and DNA/protein and RNA/protein ratios in our system.

After adding non-nutrient salt medium to growing microplasmodia the DNA content increases until about 8 hours, after a lag period of about 4 hours, and decreases gradually after that (figure 24). The RNA and protein contents decrease during the first three hours, increase until about 8 hours and then decrease gradually (figure 24). The DNA/protein ratio increased until about 10 hours and decreased after that. The RNA/protein ratio decreased gradually during the

varying from experiment to experiment. The RNA/protein ratio increased after about 10 hours until about 30 hours and decreased a little after that (figure 28).

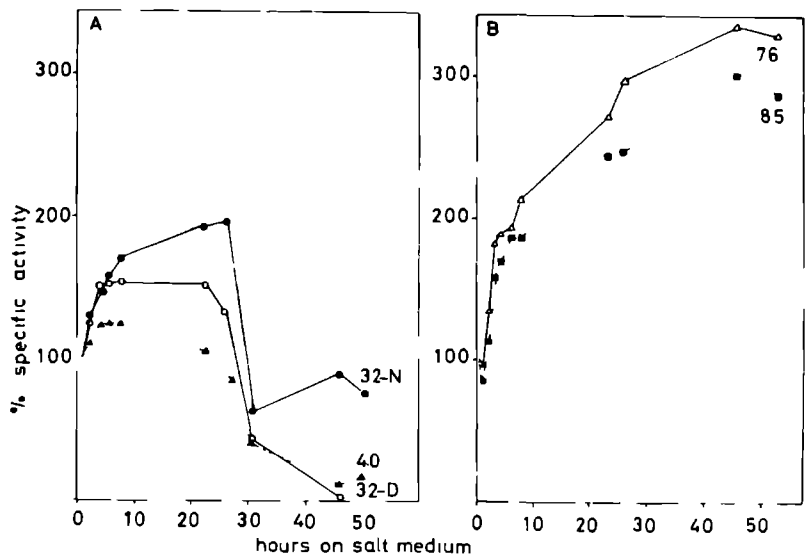


Figure 26. Specific activities of DNA degrading enzymes during sphérulation.
The data are expressed as percentage of the specific activity of growing microplasmodia at the time starvation was initiated (zero time).

Figure 29 presents the changes of the specific activities of the DNA degrading enzymes during germination of the spherules. The acid activities increased 4 - 12 fold (figure 29A), but the alkaline activities decrease sharply, remain low until about 25 hours and increased afterwards (figure 29B).

spherulation (figure 25).

The fluctuations of the specific activities of the DNA degrading enzymes during spherulation are presented in figure 26. After adding salt medium to the microplasmodia the specific activities increased during about 6 hours, then leveled off just at the moment of maximum DNA, RNA and protein content in the starved cultures (see figure 24). The alkaline activities increased the first five hours much more than the acid activities during the same time.

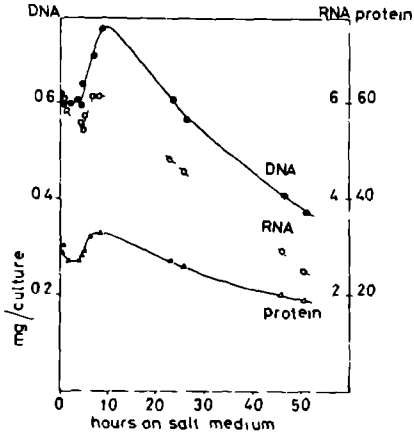


Fig. 24

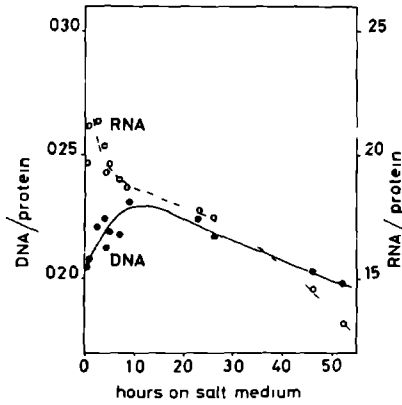


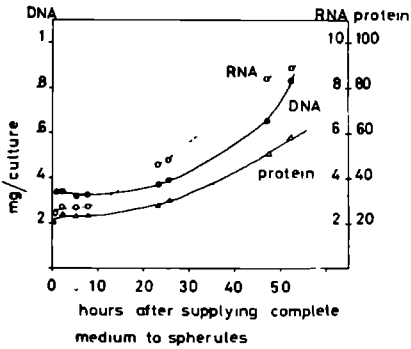
Fig. 25

Figure 24. DNA, RNA and protein content per 20 ml shaken liquid culture of starving microplasmodia in non-nutrient salt medium.

Figure 25. DNA/protein and RNA/protein ratios during spherulation.

The acid activities remained more or less constant until about 22 hours after adding salt medium and than decreased sharply (figure 26A). The alkaline activities, however, still increased during the process of spherulation (figure 26B).

After adding complete medium to microplasmodia which had been starved in non-nutrient salt medium for 60 hours, the RNA content increased after about 10 hours and the DNA and protein contents after about 20 hours (figure 27). The DNA/protein ratio decreased until about 10 hours and started to increase between 30 and 60 hours



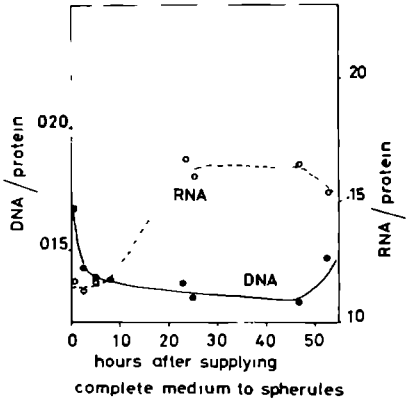


Fig. 27

Fig. 28

Figure 27. DNA, RNA and protein content per 20 ml shaken liquid culture after adding complete medium to microplasmodia which had been starved during 60 hours.

Figure 28. DNA/protein and RNA/protein ratios during germination of the spherules.

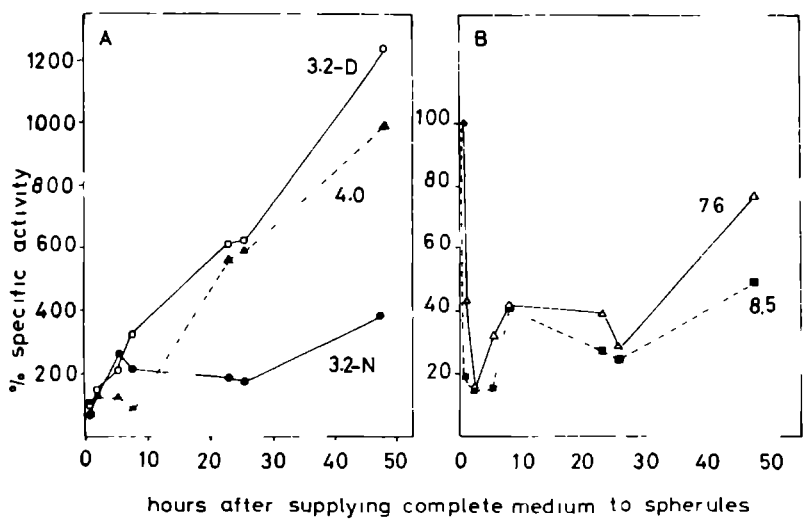


Figure 29. Specific activities of DNA degrading enzymes during germination of spherules (microplasmodia which had been starved during 60 hours (zero time)).
A. acid activities, B. alkaline activities.

Discussion

In the foregoing fluctuations in specific activity of DNA degrading enzymes (to be designated further as DNases) under changing physiological conditions have been demonstrated. The various fluctuations in activity are summarized in table X.

However, before trying to correlate changes of specific enzymes with specific processes, the nature of these changes should be established. As the activities were expressed as specific activities, fluctuations in protein content will simulate changes in enzyme activity, although the amount of enzyme is constant. As can be seen in figure 22A and 22B the specific activities of the acid and alkaline DNases vary in opposite directions, thus excluding changes in protein content as the sole source of the fluctuations. And indeed the total activity of the alkaline DNase per culture did decrease. During starvation the increase in specific activity of the alkaline DNases (figure 26) could be explained by the decrease in protein content (figure 24) but between 10 and 30 hours the activity per culture also increased. After 30 hours the total activity per culture decreased, but this was very likely due to incomplete destruction of the spherules by sonification. The enzyme activities were expressed as specific activities to overcome difficulties in the destruction of spherules and fluctuations due to different size of macroplasmodia and cultures of microplasmodia.

Another possible interpretation of the fluctuations in enzyme activity is the presence of varying amounts of inhibitors or activators. However, from some preliminary experiments in mixing extracts from cultures of different ages (Schönherr et al., 1970; Brent, 1973; Slor et al., 1973) we found only evidence for the existence of an inhibitor of the pH 3.2 DNase during spherulation. During the mitotic and the growth cycle no evidence until now was obtained for the existence of activators or inhibitors. But more extensive studies will be needed to obtain certainty about this.

Preliminary experiments to inhibit protein synthesis with cycloheximide (50 µM) during 60 minutes in different parts of the mitotic cycle indicate that the increase of the alkaline DNA degrading activities could be suppressed, thus suggesting that the fluctuations in activity of at least the alkaline activities could be due to de novo synthesis of enzyme molecules (Mitchison, 1971). But the presence of alkaline DNA degrading activities in all parts of the mitotic cycle

suggests the existence of stable enzyme molecules, or of permanent enzyme synthesis with fluctuations in synthetic rate.

In many other organisms enzyme fluctuations during the growth cycle have been demonstrated. In the growth cycle of Escherichia coli the different DNases exhibit different changes in specific activity (Shortman and Lehman, 1964). Also other enzymes exhibit fluctuations in activity during the growth cycle of prokaryotes (Dean and Hinshelwood, 1966), or of animal cells in culture (Ward and Plagemann, 1969). In growing microplasmodia of Physarum polycephalum Chet et al. (1973) observed fluctuations in RNase-activity.

During sphérulation and germination of the spherules we found striking differences between the acid and alkaline activities (figure 26 and 29). Changes in activity of other enzymes in Physarum polycephalum were observed during starvation on non-nutrient salt medium (see review by Hüttermann, 1973), sphérulation induced by mannitol (Hüttermann and Chet, 1971; Chet et al., 1973) and germination of the spherules (Hüttermann et al., 1970). Acidic nuclear proteins from starved cultures are different from those of growing cultures (Le Stourgeon et al., 1973). In other organisms DNase activity has been demonstrated in the acidic nuclear proteins (O'Conner, 1969).

In our system we found in many experiments a net increase in DNA content and decrease followed by an increase of the RNA and protein contents during the first 8 hours of starvation (figure 24). This is at variance with the results of Sauer et al. (1970). These authors found no net increase, but only a decrease after about 8 hours, although with radioactive precursors they could demonstrate DNA, RNA and protein synthesis, and at least for RNA no turnover of the newly synthesized RNA. The decrease of the DNA, RNA and protein contents is in Sauer's system faster than in ours. Perhaps the different behaviour of the DNA, RNA and protein contents in our system compared to the system of Sauer et al. is due to the starvation medium which in our system differs in minor details from theirs.

During the mitotic cycle very sharp and reproducible changes in alkaline specific activities were demonstrated. Total protein increases gradually during the mitotic cycle (Schiebel, 1973) so these fluctuations can not be explained by changes in protein content of the macroplasmodia.

With the acid activities no reproducible results were obtained.

Perhaps the fluctuations of the acid activities during the mitotic cycle are influenced by the same process(es) that cause(s) an increase in activity of the DNA degrading enzymes during the second part of the logarithmic phase. This is supported by the observation that the specific activities after mitosis III of the acid DNA degrading enzymes do not return to the same level as before mitosis III.

Several other enzymes in the slime mold Physarum polycephalum show periodic increases in activity during the mitotic cycle (see review by Schiebel, 1973). For some of them, ribonuclease (Braun and Behrens, 1969) and thymidine kinase (Hildebrandt and Sauer, 1973), strong indications exist that these fluctuations are due to de novo synthesis of enzyme molecules.

Table X. Fluctuations of DNase specific activities, DNA/protein and RNA/protein ratios during growth and differentiation.

specific activity	growth cycle	sphérulation	spherule germination
at acid pH	increase	incr. - decr.	increase
at alkaline pH	decr. - incr.	increase	decrease
DNA/protein	decrease	incr. - decr.	decrease
RNA/protein	incr. - decr.	decrease	increase

Table X, summarizing the results presented shows clearly that the acid DNases as a group behave in a way different from the alkaline group and that within a group - notwithstanding small differences - the enzymes change in the same way. The different behaviour of the acid and alkaline activities during different physiological conditions suggests strongly that they are involved in different mechanisms regulating the differentiation process.

Many authors suggest that the DNases are related to DNA synthesis mostly because they found high DNase activity during DNA synthesis (see review by Lehman, 1967; Koerner, 1970; Lesca, 1971). We also found sharply increasing alkaline DNase activities during the first part of the S-phase (figure 23) suggesting a relationship with the synthesis of DNA. The sharp increase during the first 8 hours of starvation showed the same (figure 26). However, the maxima in specific

activity of DNA degrading enzymes in other parts of the mitotic cycle, the further increase of the alkaline activities during the starvation process after 10 hours and the increase of all activities during the last part of the logarithmic phase, suggest that DNA degrading activities are also involved in processes other than DNA synthesis in Physarum polycephalum.

Part of the DNases could play a role in RNA synthesis, as was already suggested by Vogt (1969). During the mitotic cycle two maxima in RNA synthesis are observed, namely during the S-phase and during the mid G-phase (Schiebel, 1973). As table X shows the fluctuations in acid DNase activity are mainly correlated to the changes in RNA/protein ratio and the fluctuations in alkaline DNase activity are chiefly correlated to the changes in DNA/protein ratio.

One would not expect, however, DNases which play a role in DNA synthesis, to possess a high degradative activity in vivo. Therefore, DNases mainly involved in nucleic acid catabolism - e.g. of foreign nucleic acids ingested by phagocytosis (Gray and Alexopoulos, 1968) - with high activities in vivo could easily overshadow the activity of specific DNases. In chapter V was described already that the different DNase activities consisted of several isoenzymes.

It remains still uncertain, how far the activities measured in vitro are a true measure for the activities in vivo. During the preparation of the extracts losses or inactivations of inhibitors or activators could occur; enzymes contained within impermeable membranes in cell organelles could be set free, etc. Therefore further studies on these enzymes, e.g. on their cellular localization, on the characteristics of purified enzymes, their behaviour towards inhibitors of nucleic acid and protein synthesis will be needed to give a clearer insight into the cellular processes they are involved with.

THE ISOLATION OF NUCLEI FROM THE SLIME MOLD PHYSARUM POLYCEPHALUM. COMPARISON OF TWO ISOLATION MEDIA

Introduction

When we started to isolate nuclei from Physarum polycephalum only one published method for the isolation of nuclei of the slime mold Physarum polycephalum existed, namely the method of Mohberg and Rusch (1964).

In our hands this method yielded nuclear preparations which were rather contaminated with non-nuclear material as determined with phase contrast microscopy. Furthermore, there is evidence that isolation media containing sucrose injure chromatin structures, probably because of their low ionic strength (Chevaillier, 1971). Polytene chromosome structure of Drosophila hydei is damaged by sucrose media (Berendes, personal communication).

We thought it necessary therefore to make a critical study of the isolation of nuclei from Physarum polycephalum. On the one hand several manipulative steps (such as homogenization, number and mode of centrifugations, centrifugal force) were tested; on the other hand the chemical composition of the isolation media used was varied. Finally, a number of parameters of the nuclei isolated by the methods proven best were compared as the properties of the nuclei may depend on the isolation medium.

Materials and methods

1. The isolation of nuclei

a. Preliminary method. Adapted from Mohberg and Rusch (1964).

Microplasmodia were collected from full-grown 20 ml shaken liquid cultures by centrifugation during 2 min. at 500 g and washed quickly twice with ice-cold aqua bidest. The pellet of one culture was divided into two equal parts, and each part suspended in 20 ml homogenisation medium. Homogenisation was achieved with five up and down strokes in a teflon-glass Potter-Elvehjem homogeniser combined with a Tri-R Stir-R model S 63C motor set at stage 3.5. Unbroken microplasmodia, slime and other material were removed by centrifugation at 90 g during 5 min. Nuclei were

pelletted from the supernatant and washed at 1500 g during 15 min., unless otherwise stated. Nuclear pellet was suspended in 20 ml wash medium and the number of nuclei and of non-nuclear particles and material were determined in a hemocytometer under a phase contrast microscope (Wild 20509) set at 480 x magnification.

b. Final method. Adapted from Mohberg and Rusch (1971).

This method is described in detail under chapter II (Materials and general methods).

2. Characterization of the nuclear preparations

a. Phase contrast and electron microscopy were carried out as described under chapter II.

b. Determinations of DNA, RNA and protein content of the nuclear pellets were carried out as described under chapter II.

c. To measure the RNA synthetic activity of the nuclear pellets microplasmodia were labelled during one night with 0.02 µCi ^{14}C-methyl-thymidine (NEN Radio-chemicals) per ml growth medium and nuclei were isolated. Nuclear pellets from 4 shaken liquid cultures were suspended in 3.5 ml nuclear isolation medium, 0.1 ml ribonucleotide triphosphates (adenine, uridine, cytosine and guanine) (1 mM in aqua bidest.) and 0.1 ml 5-^{3}H-uridine-triphosphate (100 µCi/ml aqua bidest.; specific activity: 15.9 Ci/mM; NEN Radio-chemicals) were added. Incubation was carried out at 30°C with shaking in a waterbath and after various times 0.5 ml aliquots were pipetted from the total sample in tubes at 0°C containing 0.2 ml RNA-solution (2 mg/ml aqua bidest.; yeast RNA, Schwarz Bioresearch Inc.). Tubes were shaken and 1.0 ml 10% (w/v) CCl_3COOH containing 0.02 M $Na_4P_2O_7$ was added. Tubes were shaken vigorously and after 10 minutes were filtered over glassfiber filters (Joymar Scientific Inc.) by suction. Filters were washed three times with 15 ml 0.5 N $HClO_4$, three times with 15 ml 96% ethanol-ether (3 : 1) and once with 0.5 N $HClO_4$. The filters were put in counting vials, 0.5 ml 0.5 N $HClO_4$ was added and after 60 minutes at 70°C 10 ml liquid scintillation solution (4 g Omnifluor in 1300 ml Triton - toluene (v : v = 23 : 27) were added. Radioactivity was determined in a Philips Liquid Scintillation Counter.

d. SDS - polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969); gels contained 10% (w/v) acrylamide

instead of 7.5%. Nuclei and microplasmodia were precipitated with 5% CCl_3COOH, washed twice with 96% ethanol, once with ether and dried. Samples were dissolved in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% (w/v) SDS and 1% (w/v) β-mercaptoethanol. After storage for two hours at 37°C the preparation was heated to 100°C for 3 minutes and electrophoresis was carried out.

Results

1. The isolation of nuclei

When we started to isolate nuclei from the slime mold Physarum polycephalum according to Mohberg and Rusch (1964) we obtained nuclear preparations severely contaminated under the phase contrast microscope with non-nuclear particles: many great slimy clumps to which numerous nuclei were adhering were visible (see figure 30).

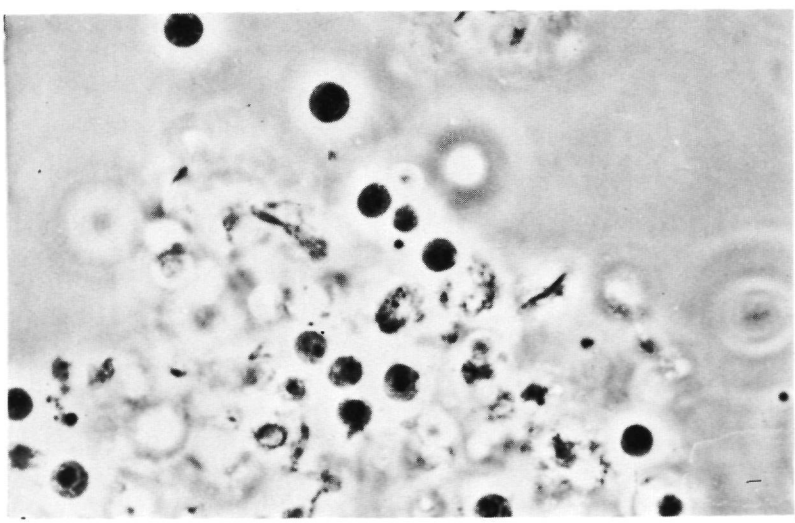


Figure 30. Phase contrast photomicrograph of a slimy clump in a nuclear preparation in salt medium. x 1300.

We set out then to try to improve the yield and the purity of the nuclei isolated. Several procedures successful to other materials

Table XI. Survey of the nuclear isolation methods applied to Physarum polycephalum.

No.	Homogenization medium	Purification I	Purification II	% of nuclei relative to method 1	% non-nucl. particles relative to method 1	References
1	sucrose medium	sucrose medium 3x washing	–	100	100	a
2	salt medium	salt medium 3x washing	–	112	84	–
3	.25 M sucrose + 3 mM $CaCl_2$	sucrose-$CaCl_2$ 3x washing	–	97	very high	b
4	10 mM Tris/HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$	.25 M sucrose, 3 mM $CaCl_2$ 3x washing	–	21[1)]	133	b,c
5	0.1% Triton X-100, 0.001% spermine	.25 M sucrose, 3 mM $CaCl_2$ 3x washing	–	96	very high	d
6	1.5% citric acid	.25 M sucrose, 3x washing	–	no nuclei visible	–	e
7	0.5 M sucrose, 3 mM $CaCl_2$, 10 mM KCl, 10 mM Tris/HCl, pH 7.0	1.5 M sucrose on 2.0 M sucrose + 3 mM $CaCl_2$ 45.000 g, 30 min.	.25 M sucrose, 3 mM $CaCl_2$ 3x washing	80	very high	f
8	salt medium	as method 7	salt medium 3x washing	75	140	f,g
9	salt medium	0-10% Ficoll in salt medium	salt medium 3x washing	no nuclei visible[2)]	–	b,g

1) nuclei optically empty 2) some nuclei were seen, but they were badly swollen

References: a. Mohberg and Rusch, 1964; b. Allfrey and Mirsky, 1957; c. Rich, 1967; d. Prescott, 1966; e. Dounce, 1955; f. Stern, 1967; g. Boyd et al., 1968.

were tried, such as repeated washing, or centrifugation through high density sucrose. Furthermore, a number of changes in the composition of the isolation medium were tried, such as addition of $CaCl_2$ or spermine. Also simple media such as Tris buffers or citric acid solutions were tested. For the sake of brevity all variations tested are summarized in Table XI.

From these data it can be concluded that procedures giving good results when applied to other materials yielded worse nuclear preparations than the method of Mohberg and Rusch (1964) when they were applied to the slime mold Physarum polycephalum.

Our first aim then was to improve on the method of Mohberg and Rusch (1964) to obtain purer preparations. A very important step was the homogenization. This must be carried out with excess of isolation medium, about 200 ml per microplasmodial pellet from one shaken liquid culture. Contaminations once pelletted with the nuclei cannot be removed, they must be removed before pelletting the nuclei. Therefore initially the homogenate, after centrifugation at 90 g during 5 minutes to remove unbroken microplasmodia, slime and other particles, was filtered over plankton gauze with 100, 63 and 31 μ pore diameter. This yielded good preparations from which most of the slime clumps were removed.

During these studies Mohberg and Rusch (1971) published a new paper describing details of their isolation method. They also remarked that an excess of homogenization medium was needed during the homogenization and that the homogenate should be filtered. They filtered their homogenates over milk filters. By using these filters we obtained still better preparations compared to plankton gauze. Nearly all "slimy fields" were removed (less than 1% of the nuclei).

Still purer preparations - as judged by the number of particles smaller than nuclei - can be obtained by layering the filtrate over 1 M sucrose according to Mohberg and Rusch (1971). However, the recovery of nuclei becomes much lower and the time needed for isolation is increased, as was also remarked by Bradbury et al. (1973).

2. Partial characterization of nuclei prepared according to the procedure of Mohberg and Rusch in different media

This part of the study was restricted to a comparison of nuclei isolated according to the Mohberg and Rusch (1971) procedure with the original sucrose medium, respectively with the new salt medium.

As the properties of nuclei may depend on the isolation medium, we wanted to have more than one isolation medium at our disposal.

Figure 31 and 32 show phase contrast pictures of nuclei in salt or in sucrose medium. Salt nuclei were a little more swollen than sucrose nuclei, the diameter was about 1 μ greater than that of the sucrose nuclei (see table XII). The diameter of nuclei in smear preparations is 5.35 ± .23 μ, as can be seen in figure 9 in the paper of Guttes et al. (1961). The optical density under phase contrast of salt nuclei was lower than that of sucrose nuclei.

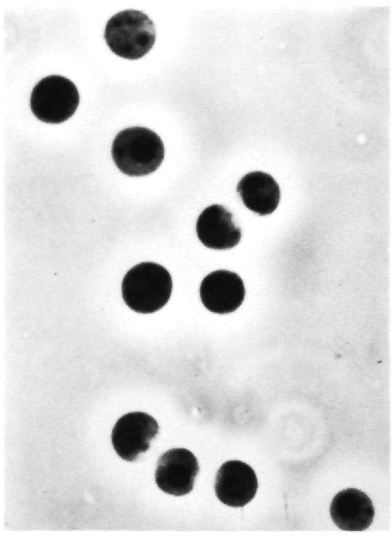


Fig. 31

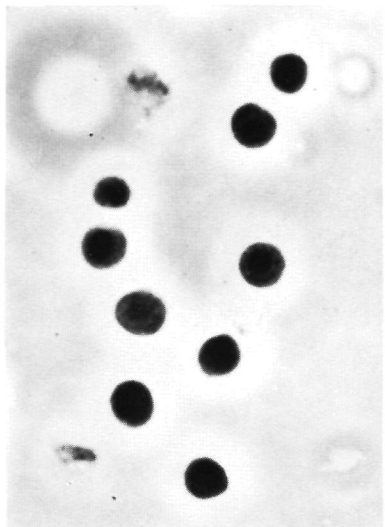


Fig. 32

Figure 31. Phase contrast micrograph of salt nuclei. x 1600

Figure 32. Phase contrast micrograph of sucrose nuclei. x 1600

Percentage of non-nuclear material in salt medium preparations was in all experiments lower than in sucrose preparations, although this percentage varied from experiment to experiment (table XII). Percentage of nuclei with fragmented nucleoli was equal in both preparations. Recovery of number of nuclei varied from 40 - 60%. As judged by the phosphatase activity of the nuclear preparations cytoplasmic contamination was very low (table XIII).

Table XII. Appearance of nuclei under phase contrast.

Exp.no.	medium	diameter in µm	% nuclei with fragmented nucleoli	% non-nuclear material
1	salt	5.79 ± .05(45)*	33	
	sucrose	4.78 ± .06(45)	32	
2	salt		37	34
	sucrose		37	41
3	salt		31	60
4	salt			82
	sucrose			157
5	salt (sample 1)			29
	salt (sample 2)			31
	sucrose (sample 1)			53
	sucrose (sample 2)			60

* average ± standard deviation (number of nuclei)

Figure 33 shows an electron micrograph of a salt nucleus. These nuclei have essentially the same appearance as sucrose nuclei in the electron microscope as can be seen e.g. in figure 2 of the paper of Mohberg and Rusch (1971). Nucleolus and chromatin can clearly be distinguished. A nuclear membrane is not visible probably due to lipid and protein extraction from the membrane by using the detergent Triton X-100 (Comings and Okada, 1970). In "whole mount" preparations of these nuclei annuli are observed (Schel and Wanka, 1973). This suggests that the nuclear membrane is not completely removed.

Table XIV presents the chemical composition of the nuclei. DNA per nucleus, protein/DNA and RNA/DNA ratios of the sucrose nuclei

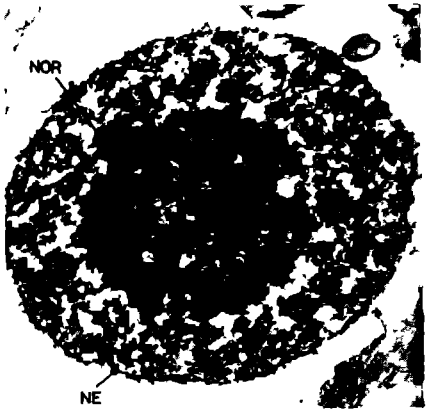


Figure 33. Electron micrograph of a salt nucleus. x 19.000.

Nu : nucleolus
C : chromatin
NOR: nucleolar organizer region
S : slime

Table XIII. Recovery of nuclei from microplasmodia.

Exp.no.	medium	hemocytometer counts 1) %	DNA content 2) %	phosphatase recovery 3) %
2	salt	66		
	sucrose	63		
4	salt	47	45	
	sucrose	31	43	
5	salt (sample 1)	43	43	0.53
	salt (sample 2)	61	61	0.77
	sucrose (sample 1)	49	41	0.61
	sucrose (sample 2)	47	41	0.73

1) Nuclei were counted in the crude homogenate (100%) and in the final nuclear preparation.

2) DNA content was measured in a portion of the microplasmodia (100%) and in the final nuclear preparation.

3) Phosphatase activity was measured according to Hüttermann et al. (13) in microplasmodia (100%) and in nuclear preparations.

Table XIV. Chemical composition of the nuclear preparations.

isolation medium	pg DNA/nucleus*	protein/DNA**	RNA/DNA**
salt	.95 ± .05 (6)[1]	4.47 ± .38 (5)	.53 ± .05 (5)
sucrose	1.19 ± .06 (6)	5.99 ± .91 (5)	1.12 ± .16 (5)

1) average ± standard deviation (number of independent experiments)

* based on the number of nuclei counted in the hemocytometer

** based on the total preparations

Table XV. Relative recovery of nuclei per 20 ml liquid shaken culture.

isolation medium	nuclei/culture	DNA/culture
salt	1.40 ± .08 (3) [1]	1.12 ± .08 (3)
sucrose	1	1

1) average ± standard deviation (number of independent experiments)

Recovery of sucrose nuclei was chosen as reference. Relative recoveries were chosen instead of absolute recoveries because the total amount per culture varied much from experiment to experiment (93 - 490 x 10^6 nuclei/culture and 115 - 420 µg DNA/culture in the experiments of table XV) depending on the moment the culture was harvested.

were equal to the data presented by Mohberg and Rusch (1971). Salt nuclei possessed lower protein/DNA and especially much lower RNA/DNA ratios than sucrose nuclei. According to hemocytometer counts salt medium gives a higher recovery than sugar medium; as measured by DNA content both methods are equal (table XV).

Figure 34 shows that both nuclear preparations are capable to incorporate ^{3}H-UTP in material which is acid-insoluble, sensitive to RNase and alkali, although in sucrose nuclei with a higher synthetic rate. Very striking was that in all preparations from the same sample of microplasmodia, in sucrose nuclei about ten times more radioactive label was attached to macromolecular material than in salt nuclei at zero time (see figure 34).

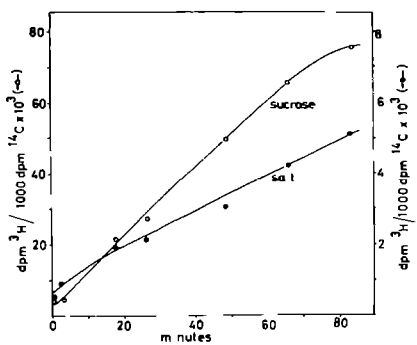


Figure 34. Incorporation of ^{3}H-uridine triphosphate in acid-insoluble material by salt and sucrose nuclei.

Figure 35 shows densitometer scans of SDS-polyacrylamide gels containing total nuclear proteins of sucrose and salt nuclei. Only small quantitative differences were observed between the two preparations. But all bands could be seen in both samples.

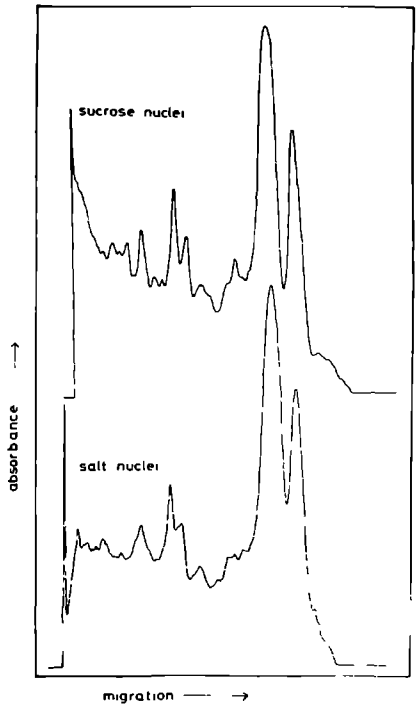


Figure 35. Densitometer scans of the proteins of sucrose and salt nuclear preparations separated electrophoretically according to Weber and Osborn (1969).
Gels were scanned on a Kipp Densitometer DD_2 with filter C (transmission range 500 - 550 nm).

Discussion

The results presented suggest strongly that the isolation procedure according to Mohberg and Rusch (1971) yielded so far the best nuclear preparations.

However, it remains unclear which nuclear isolation medium provides nuclei which most resemble nuclei in vivo. Under phase contrast microscopy the preparations of salt nuclei contain less morphologically non-nuclear material, and their protein/DNA and RNA/DNA ratios are lower than those of sucrose nuclei.

But the lower protein/DNA and RNA/DNA ratios do not necessarily prove higher purity. Losses of nuclear proteins and RNA relative to DNA could well account for these differences. The amount of (cytoplasmic) phosphatase is the same in both preparations (table XIII). If the content of this enzyme really was an indication of overall cytoplasmic contamination, both preparations have the same purity, and losses of protein and RNA from nuclei in salt medium relative to sucrose medium have occurred.

From the SDS-polyacrylamide gels, however, no indications were obtained that specific proteins are lost from salt nuclei compared to sucrose nuclei.

Both preparations are able to incorporate ^{3}H-UTP in RNA like material. The different incorporation velocities could be due to the different composition of the nuclear isolation media in which the incubations were carried out. However, some loss of RNA polymerase from the salt nuclei cannot be ruled out. Conversely, this lower activity may be interpreted as an indication that proteins repressing RNA synthesis, e.g. RNases are not lost from these nuclei. In chinese hamster cell nuclei isolated in sucrose medium strong indications are present that many of the non-histone proteins of these nuclei are of cytoplasmic origin (Comings and Tack, 1973). Probably a higher aspecific adsorption of ^{3}H-UTP occurs in the sucrose nuclear preparations (see figure 34). This phenomenon was, however, not studied in detail.

There is a discrepancy in the recovery of nuclei as determined by hemocytometer counts or through determination of DNA (table XV). The latter is consistently higher in sucrose media, which would result in a higher DNA content per nucleus (cf. table XIV). This does not, however, constitute proof of a loss of DNA from salt nuclei. From table XII it can be concluded that the fraction of particles which are

not morphologically intact nuclei is higher in sucrose nuclear preparations. The lower recovery of morphologically intact nuclei in sucrose media, coupled to equal recovery of DNA reactive material could as well be explained by a greater fragmentation of nuclei in sucrose media. It is moreover very well possible that these nuclear fragments are responsible for the high rate of incorporation of ^{3}H-UTP (see above).

Bradbury et al. (1973) showed recently that to obtain good preparations of the various subnuclear components, e.g. chromatin and nucleoli, sucrose media of different composition and ionic strength were obligatory. Our salt medium was very convenient to obtain whole mount preparations of nuclei for the electron microscopic observation of chromatin structure of Physarum polycephalum (Schel and Wanka, 1973).

As every isolation method and medium introduces specific artefacts (Dounce and Ickowics, 1969), the salt medium presented here might be a good alternative for the sucrose medium to study the biochemical and morphological composition of the nuclei of Physarum polycephalum.

Acknowledgements

I wish to thank Drs. J.W.M. Derksen for stimulating discussions, Dr. A.M. Stadhouders for performing the electron micrograph, Drs. J.H.N. Schel and Dr. C. Stumm for aid and advice with the phase contrast micrographs and Drs. L.H.F. Mullenders for performing the electrophoretic separations.

CHAPTER VIII

DNA DEGRADING ACTIVITIES IN NUCLEI FROM PHYSARUM POLYCEPHALUM

Introduction

DNases are supposed to play a role in many processes which are located in the cell nucleus, e.g. DNA replication and transcription or DNA repair (see reviews by Lehman, 1967 and Lesca, 1971). So the occurrence of DNases in nuclei is very likely and many authors have indeed demonstrated in nuclei the existence of DNases.

Very early Brown et al. (1952) already reported the presence of DNase II in the nucleus. Endonucleases were demonstrated in the nuclei from rat liver (O'Conner, 1969; Burgoyne et al., 1970; Baril et al., 1971), from mouse liver (Hewisch and Burgoyne, 1973), from HeLa cells (Studzinsky and Churchill, 1971) and from Paracentrotus lividus embryo's (Parisi and De Petrocellis, 1972). Exonucleases were demonstrated in nuclei from rabbit bone marrow (Lindahl et al., 1969). Churchill et al. (1973) could separate the DNases from HeLa nuclei in four enzymes on CM-cellulose. With a histochemical method Swingle and Cole (1964) demonstrated the existence of DNase II in rat liver nuclei. Lesca (1968) in comparing DNase II in mouse liver cells with cytochrome oxidase showed that the DNase II activity in nuclei was not a lysosomal contamination and Slor and Lev (1971) in comparing DNase II in calf thymus cells with acid phosphatase concluded that DNase II activity is partly localized in the nucleus. With ^{14}C-DNase II it was demonstrated that DNase II did not bind preferentially to nuclei during the isolation procedure of calf thymus nuclei (Slor, 1973).

Most of these nucleases in nuclei were demonstrated by the formation of acid-soluble products from DNA after incubation with homogenates from isolated nuclei (Slor and Lev, 1971; Parisi and De Petrocellis, 1972, Churchill et al., 1973).

Nuclei from Physarum polycephalum are also capable to synthesize DNA and RNA (Grant, 1972; Schiebel, 1973). So probably these nuclei do contain DNases and experiments were undertaken to demonstrate DNA degrading activities in the nuclear preparations.

To establish the DNase activities in the nuclear preparations as true nuclear enzymes, the activities were compared to the activity of

acid phosphatase.

In mammalian tissues acid phosphatase is localized in the cytoplasm and mainly associated with the lysosomes (De Duve et al., 1962). In Physarum flaviconum acid phosphatase is chiefly localized in food vacuoles and dictyosomes (Kazama and Aldrich, 1972) and in Dictyostelium discoideum in food vacuoles and the cell surface (Gizelius, 1971). Occasionally occurrence of phosphatase activity in nuclei was considered to be an artefact by many authors (Holt, 1959; Deane, 1963; Sexton et al., 1971; Gizelius, 1971; Kazama and Aldrich, 1972).

Materials and methods

Nuclei were isolated as described in chapter II. Nuclear pellets were dispersed in homogenisation buffer, ultrasonicated and centrifugated as described under chapter II. After sonification no intact nuclei were visible under the phase contrast microscope, although nucleoli and other particles could be observed.

DNA degrading activities were measured as described under chapter II. Acid phosphatase activities were measured according to Hüttermann et al. (1970).

Results

The known DNA degrading activities in crude enzyme extracts had in isolated nuclei low specific activities compared to the specific activities in the crude enzyme extracts (table XVI). So probably these enzymes are not localized in the nucleus.

Table XVI. Specific activity of the DNA degrading activities in nuclei and crude enzyme extracts from Physarum polycephalum.

enzyme	ΔE_{260} / mg protein / min x 10^3		
	crude enzyme extr.	salt nuclei	sucrose nuclei
3.2 - nat.	54.2	6.98	10.1
3.2 - den.	95.8	3.2	3.1
4.0	198.8	4.9	5.1
7.6	11.9	.52	.38
8.5	16.5	.87	1.03

Crude enzyme extract was prepared from a part of the same microplasmodia from which the nuclei were isolated.

It is, of course, possible that the DNA degrading activities in nuclear preparations possess other pH optima. However, the effect of the pH on the DNA degrading activities in the nuclear preparations was not reproducible. Highest specific activities on denaturated DNA were found around pH 3.8 - 4.0, but at lower pH's and in the alkaline range the specific activities fluctuated from experiment to experiment. With native DNA as substrate no pH optimum was observed.

To examine if this DNase were derived from the cytoplasm, the activity of acid phosphatase in the nuclear preparations was compared to the activity of DNA degrading activity in the same preparations.

Table XVII shows that from the total activities acid phosphatase is to a greater extent associated with the nuclear fraction than the DNA degrading activity at pH 4.0. The DNA degrading activity is more easily removed during the purification steps of the nuclear isolation procedure than the acid phosphatase activity.

Discussion

DNase activity with a maximum activity at pH 4.0, capable to form acid-soluble products from salmon sperm DNA was demonstrated in nuclear preparations from Physarum polycephalum. In these preparations Kuyper et al. (results to be published) showed the existence of a slow-working endonuclease, capable of making nicks in circular MS 13 phage DNA at pH 8.5.

Braun and Behrens (1969) were unable to observe RNase activity in nuclei from Physarum polycephalum. In isolated nuclei from many organisms, however, endonucleolytic (Mirault and Scherrer, 1972; Prestayko, Lewis and Busch, 1972) and exonucleolytic RNases (Lazarus and Sporn, 1967; Liau, Craig and Perry, 1968; Perry and Kelly, 1972) playing a role in the processing of ribosomal precursor RNA to ribosomal RNA are described. So lack of RNase activity in nuclei isolated from Physarum polycephalum could be due to leakage of this (these) enzyme(s) from the nuclei during the isolation procedure. Leakage could also account for the low DNase specific activities in nuclear preparations compared to crude enzyme extracts. In many organisms DNase activity was demonstrated after incubation with homogenates from isolated nuclei by the formation of acid soluble products from DNA (Slor and Lev, 1971; Parisi and De Petrocellis, 1972; Churchill et al., 1973) and in some of them, e.g. in nuclei from Paracentrotus lividus embryo's (Parisi

Table XVII. DNase activity compared to acid phosphatase activity in nuclei from Physarum polycephalum.

	total DNase activity at pH 4.0 $\Delta E_{260}/min \times 10^{-3}$		total phosphatase activity $\Delta E_{410}/min \times 10^{-3}$		DNase/phosphatase activity ratio $\times 10^{3}$		activity ratio %	
step	salt	sucrose	salt	sucrose	salt	sucrose	salt	sucrose
total homogenate	10.79	6.554	140.37	99.63	76.9	65.8	100	100
90 g - supernatant	7.450	6.007	111.46	97.25	66.8	61.8	86.9	93.9
crude nuclei	.0321	.0424	.750	.773	42.8	54.8	55.7	83.3
washed nuclei	.0174	.0191	.477	.421	36.5	45.4	47.5	69.0

and De Petrocellis, 1972) the specific activity of the DNase was in nuclear preparations higher than in total homogenates.

However, acid phosphatase which is considered to be a cytoplasmic enzyme (Deane, 1963; Gizelius, 1971; Kazama and Aldrich, 1972) was more associated with the nuclear preparations than the pH 4.0 DNase activity. So probably part or even all of this DNase activity was derived from association with cytoplasmic DNase having highest activity at pH 4.0.

This conclusion is based on three assumptions. First, acid phosphatase does not possess a preferent binding capacity to the nuclei. Secondly, acid phosphatase is neither in Physarum polycephalum localized in the nucleus. This has not been established until now. Thirdly, in nuclear preparations the DNase activity is not more inhibited than the acid phosphatase activity.

Therefore these experiments do not exclude the possibility of the existence of specific nuclear DNases, but only give evidence that a great part of the DNA degrading activities of the nuclear preparations is achieved by cytoplasmic DNase associated to the nuclear preparations. To measure the activity of a specific nuclear DNase probably more purification steps from the nuclear homogenate will be needed.

GENERAL DISCUSSION AND SUMMARY

The acellular slime mold Physarum polycephalum Schwein. possesses several DNA degrading enzymes. In crude extracts these enzymes show maximal activity at different pH's. One enzyme activity degrades native DNA with a maximum activity at pH 3.2. Four others degrade heat denaturated DNA, and have their maximum activities around pH 3.4, 4.0, 7.6 and 8.5 respectively.

These activities are affected by divalent cations in different ways. The acid activities were inhibited by Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+}; however, to different extents. Zn^{2+} inhibited the pH 4.0 activity, but stimulated the pH 3.2 activities. Also EDTA inhibited the acid activities. Probably these enzymes need the presence of specific divalent cations in the crude enzyme extracts to attain their maximal activity (chapter IV).

The activities at pH 7.6 and pH 8.5 were inhibited by Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} and EDTA. Addition of Co^{2+}, or EDTA suppressed their activity completely. On the contrary, the alkaline activities are stimulated by Mg^{2+} and Mn^{2+}. Mg^{2+} had the greatest effect on the pH 7.6 activity; 5 mM $MgCl_2$ produced the highest stimulation at pH 7.6, while at pH 8.5 already 1 mM $MgCl_2$ gave maximum stimulation. On the other hand, Mn^{2+} stimulated most the pH 8.5 activity and 5 mM $MnCl_2$ had the highest effect (chapter IV).

Crude enzyme extracts (pH 7.1) were kept at different temperatures. The enzyme activity decreased in dependency on the increasing temperature in different ways. The pH 3.2 activities, especially the pH 3.2 activity on denaturated DNA, were most thermolabile. The activities at pH 4.0 and 8.5 were most thermostable (chapter IV).

Unlike the other activities the pH 3.2 activities decreased during tne incubation, probably due to the low pH, because in the crude enzyme extract (pH 7.1) the activities were stable at 30°C. The pH 3.2 activity on denaturated DNA became inactivated most strongly during the incubation (chapter IV).

From the results mentioned above - different effect of divalent cations, different stability towards higher temperatures, different stability of the 3.2-DNases during the incubation - was concluded that

the activities consist of different enzymes (chapter IV). Only Hiramaru et al. (1969) described the existence of DNA degrading activities in slime molds. These authors showed in crude enzyme extracts of Physarun polycephalum only one DNase activity on denaturated DNA with maximum activity at pH 4.5. After purification the activity turned out to be an aspecific nuclease. However, they prepared the crude enzyme extracts in a way completely different from ours in phosphate buffer (Hiramaru et al., 1969a). We found phosphate buffers to be strongly inhibitory on the alkaline activities.

From the distribution of the five DNase activities over several cell fractions and from the different pattern of bands after electrophoretic separation of the activities more evidence was obtained that the activities belong to different enzymes (chapter V).

After removing unbroken microplasmodia and other large particles from the homogenate with centrifugation at 90 g during 5 minutes the homogenate was divided into five successive fractions, namely a 500 g pellet, a 2500 g pellet, a 25.000 g pellet, a 100.000 g pellet and a 100.000 g supernatant. The greater part of all enzyme activities was localized in the cytosol, the 100.000 g supernatant. But the DNase activities were to a greater extent associated with the particle fractions than the RNase activities (chapter V).

The distribution of the DNase activities over the four particle fractions was compared to the distribution of the RNase activities over these fractions. Namely because the RNase activity with yeast RNA as substrate possessed only one pH optimum at pH 3.8 - 4.0 (chapter V).

The distributions of the RNase activities measured at pH 3.2, 4.0, 7.6 and 8.5 were highly correlated. On the other hand, the distributions of the DNase activities correlated badly compared to the RNase activities, except the distributions of the DNase activities at pH 7.6 and 8.5 which correlated very well. The distribution of the DNase activity compared to that of the RNase activity at the same pH was badly correlated, except the DNase and RNase activities at pH 4.0. The distribution of these two activities over the four cell fractions was highly correlated (chapter V).

After electrophoretic separation of the proteins of the crude enzyme extract on polyacrylamide gels, DNase activity in the gels was detected according to Boyd and Mitchell (1965) and the RNase activity according

to Wilson (1969).

After incubation of the gel at the acid pH's 4 - 5 bands were visible, at the alkaline pH's 7 - 9 bands. The relative intensity of the bands differed between the various activities. The RNase activities showed only two bands at any pH. However, after incubation of the gel at pH 7.6 or pH 8.5 one of the bands was hardly visible (chapter V).

From the cell fractionation experiments and from the electrophoretic separation of the enzymes it was concluded that the DNase activities consisted of a mixture of different enzymes with maximum activity at different pH's.

This conclusion was supported by the data obtained by the chromatographic separation on DEAE-cellulose columns of the acid-soluble products from salmon sperm DNA after incubation with crude enzyme extracts. At every pH mixtures of mono- and oligonucleotides were obtained. The activities at the various pH's could not be divided into exo- or endonucleases. Probably every activity consisted of mixtures of these two types of enzymes (chapter V).

The behaviour of the DNase activities under changing physiological conditions was studied. The acid DNases as a group behave in a way different from the alkaline DNases (chapter VI).

1. In growing microplasmodia in shaken cultures the specific activities of the acid DNases increased after about 25 hours after inoculation. The specific activities of the alkaline DNases decreased during the first 10 hours after inoculation, remained more or less constant until about 25 hours and increased after that.
2. The alkaline activities showed during the mitotic cycle 3 maxima in specific activity, namely during the early S-phase (0 - 1 hour after mitosis), the late S-phase (2 - 3 hours after mitosis) and the mid G-phase (5 - 7 hours after)mitosis). Although in some experiments the acid activities showed maxima in specific activity, these maxima were not reproducible. They were not observed in all experiments.
3. During spherulation the DNase specific activities increased until about 10 hours after the start of the starvation, the acid activities remained more or less constant until about 30 hours of starvation and decreased afterwards. The alkaline specific activities remained increasing during the spherulation.
4. After supplying complete medium to spherules the acid specific activ-

ities increased, the alkaline specific activities, however, decreased and started to increase only at about 25 hours after germination.

The fluctuations of the DNase specific activities were correlated with the fluctuations in the DNA and RNA contents relative to the protein contents in the shaken cultures during the different physiological conditions. The DNase activities could be connected with the breakdown of nucleic acids entering the cell for example by phagocytosis, or with the synthesis of RNA and DNA. Firm conclusions, however, could not be drawn from these data (chapter VI).

We tried to demonstrate DNase activities in nuclei isolated from microplasmodia of Physarum polycephalum. Previously, however, the nuclear isolation method was studied and several nuclear isolation methods were compared. The procedure of Mohberg and Rusch (1971) showed to yield best nuclear preparations as judged by phase contrast microscopy of these preparations. However, an isolation medium containing only salts was in some cases preferable to the sucrose medium of Mohberg and Rusch. As every isolation medium causes artefacts the salt medium could be used as a good alternative for the sucrose medium (chapter VII).

Nuclear preparations from Physarum polycephalum possessed DNase activity with maximum activity at pH 4.0. The activities, however, differed from experiment to experiment. The specific activities of the DNases in the crude enzyme extract were 5 - 50 times higher than in the nuclear preparations. The fraction of total acid phosphatase activity in the nuclear preparations was higher than the fraction of total DNase activity in these preparations. Probably the DNase activity of the nuclear preparations was mainly brought about by contamination of these preparations with cytoplasmic DNases (chapter VIII).

ALGEMENE DISCUSSIE EN SAMENVATTING

De acellulaire slijmzwam Physarum polycephalum Schwein. bezit verschillende DNA afbrekende enzymen. In ruwe extracten bleken deze enzymen bij verschillende pH's hun maximale werking te vertonen. Bij pH 3,2 was één maximale enzymaktiviteit op natief DNA. Met hittegedenatureerd DNA als substraat worden maximale aktiviteiten gevonden rond pH 3,4, 4,0, 7,6 en 8,5.

Deze aktiviteiten werden op verschillende wijzen beïnvloed door tweewaardige kationen. De zure aktiviteiten werden, hoewel in verschillende mate, geremd door Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} en Co^{2+}. Zn^{2+} remde wel de pH 4,0 aktiviteit, maar stimuleerde zeer sterk beide pH 3,2 aktiviteiten. EDTA remde eveneens de zure aktiviteiten. Vermoedelijk hebben deze enzymen in het extract aanwezige tweewaardige ionen nodig voor een optimale funktionering (hoofdstuk IV).

De alkalische aktiviteiten, d.w.z. bij pH 7,6 en 8,5, werden geremd door Ca^{2+}, Cu^{2+}, Zn^{2+} en Co^{2+} en EDTA; Co^{2+} en EDTA onderdrukten hun werking volkomen. Zij werden daarentegen gestimuleerd door Mg^{2+} en Mn^{2+}. Mg^{2+} stimuleerde vooral de pH 7,6 aktiviteit en wel maximaal in een concentratie van 5 mM. Mn^{2+} daarentegen stimuleerde het sterkst de pH 8,5 aktiviteit, en het meest in een concentratie van 5 mM (hoofdstuk IV).

Wanneer het ruwe enzymextract (pH 7,1) op verschillende temperaturen gebracht werd, werden de enzymen in afhankelijkheid van de temperatuur met verschillende kinetiek geïnactiveerd. De meest temperatuurgevoelige enzymen waren de pH 3,2 aktiviteiten en vooral de pH 3,2 aktiviteit op gedenatureerd DNA. De aktiviteiten bij pH 4,0 en 8,5 waren het stabielst onder hoge temperaturen (hoofdstuk IV).

Onder de incubatieomstandigheden waren de pH 3,2 aktiviteiten i.t.t. de andere aktiviteiten niet stabiel. Waarschijnlijk als gevolg van de lage pH, want in het ruwe extract (pH 7,1) waren de pH 3,2 aktiviteiten stabiel bij 30°C. De pH 3,2 aktiviteit op gedenatureerd DNA werd tijdens de incubatie echter sneller geïnactiveerd dan de aktiviteit op natief DNA (hoofdstuk IV).

Uit de hierboven genoemde gegevens - verschillende invloed van tweewaardige ionen, verschillende gevoeligheid voor temperaturen en ver-

schillende stabiliteit van de pH 3,2 DNase onder incubatieomstandigheden - werd de conclusie getrokken dat de aktiviteiten uit van elkaar verschillende enzymen bestonden (hoofdstuk IV).

De bevinding van meerdere pH-optima voor DNase aktiviteit in ruwe enzymextracten van Physarum polycephalum is in tegenspraak met de gegevens van Hiramaru et al. (1969a), die als enigen DNase aktiviteiten beschrijven in slijmzwammen. Deze auteurs vonden slechts één pH-optimum met gedenatureerd DNA als substraat bij pH 4,5. Mogelijk is dit een gevolg van de totaal verschillende wijze waarop zij hun enzympreparaat in fosfaat buffer bereiden. Wij vonden dat fosfaat buffers de alkalische DNase aktiviteiten sterk remden.

Een sterkere aanwijzing voor het verschillend zijn van de vijf aktiviteiten werd verkregen uit de verdeling van de DNase aktiviteiten over diverse celfracties en uit het verschillende electroforetische gedrag van deze aktiviteiten (hoofdstuk V).

Na verwijdering van ongehomogeniseerde microplasmodia en grotere brokstukken uit celhomogenaten door middel van centrifugatie bij 90 *g* gedurende 5 minuten werd het homogenaat verdeeld in vijf successieve celfracties, te weten een 500 *g* sediment, een 2500 *g* sediment, een 20.000 *g* sediment, een 100.000 *g* sediment en een partikelvrije 100.000 *g* supernatans.

De verdeling van de DNase aktiviteiten bij de verschillende pH's over de vier partikel-fracties werd vergeleken met de verdeling van de RNase aktiviteiten bij deze pH's, over deze vier fracties. Omdat gebleken was dat de RNase aktiviteit met gist RNA als substraat slechts één pH-optimum bij pH 3,8 - 4,0 bezat (hoofdstuk V).

De verdelingen van de RNase aktiviteiten gemeten bij pH 3,2, 4,0, 7,6 en 8,5 waren onderling zeer hoog gecorreleerd. De verdelingen van de DNase aktiviteiten over de fracties was vergeleken met de RNase aktiviteiten onderling lager gecorreleerd, behalve de verdelingen van de DNase aktiviteiten bij pH 7,6 en 8,5 die onderling sterk gecorreleerd waren. De verdeling van de DNase aktiviteit was slecht gecorreleerd met die van de RNase aktiviteit bij de diverse pH's, behalve de DNase en RNase aktiviteit bij pH 4,0. De verdelingen van deze aktiviteiten over de vier celfracties correleerden onderling zeer goed.

Na electroforetische scheiding van de eiwitten van het ruwe enzymextract over een polyacrylamide gel werd de DNase aktiviteit in de gel

aangetoond volgens Boyd en Mitchell (1965) en de RNase aktiviteit volgens Wilson (1969).

De zure DNase aktiviteiten bleken 4 - 5 banden te vertonen en de alkalische aktiviteiten 7 - 9 banden. De banden van de verschillende aktiviteiten waren verschillend in intensiteit. De RNase aktiviteiten vertoonden bij alle pH's 2 banden, hoewel na incubatie van de gels bij pH 7,6 of 8,5 één van de twee banden slechts zeer moeilijk zichtbaar was (hoofdstuk V).

Uit de verdeling van de aktiviteiten over de celfracties en uit de verschillende electroforetisch verkregen bandenpatronen ervan, werd geconcludeerd dat de DNase aktiviteiten bij de diverse pH's uit mengsels van diverse enzymen bestonden, die bij verschillende pH's hun maximale werking hadden.

Deze conclusie werd versterkt door de gegevens die verkregen werden uit de chromatografische scheiding over DEAE-cellulose kolommen van de zuur oplosbare hydrolyseprodukten na inwerking van ruwe enzymextracten op zalm sperma DNA. Het bleek, dat bij elke pH een mengsel ontstond van mono- en oligonucleotiden. Het was niet mogelijk de aktiviteiten bij de diverse pH's naar hun werkingstype te verdelen in exo- of endonucleasen. Waarschijnlijk bestond elke aktiviteit uit een mengsel van deze twee typen enzymen (hoofdstuk V).

Het gedrag van de DNase aktiviteiten onder veranderende fysiologische omstandigheden werd bestudeerd. Het bleek dat de zure DNasen zich als groep anders gedroegen dan de alkalische DNasen (hoofdstuk VI).

1. Tijdens de groei van microplasmodia in schudculturen steeg de specifieke aktiviteit van de zure DNasen ongeveer 25 uur na enting. De specifieke aktiviteit van de alkalische DNasen daalde de eerste 10 uur, bleef dan min of meer konstant tot ongeveer 25 uur en steeg dan sterk.
2. De alkalische DNasen vertoonden tijdens de mitotische cyclus 3 optima in specifieke aktiviteit, namelijk in de vroege S-fase (0 - 1 uur na mitose), de late S-fase (2 - 3 uur na mitose) en de midden G-fase (5 - 7 uur na mitose). Hoewel in sommige experimenten voor de zure aktiviteiten optima gevonden werden, waren deze niet reproduceerbaar. Niet in alle experimenten werden deze optima gevonden.
3. Tijdens inductie van de spherulatie door uithongering stegen de specifieke aktiviteiten van de DNasen sterk tot ongeveer 10 uur na het

begin van de uithongering; de zure bleven vervolgens min of meer konstant tot 30 uur uithongering en namen dan af. De alkalische specifieke aktiviteiten bleven tijdens het gehele spherulatie-proces stijgen.

4. Wanneer aan spherules kompleet voedingsmedium werd toegevoegd, stegen de zure specifieke DNase aktiviteiten sterk, de alkalische daarentegen daalden sterk en begonnen eerst na ongeveer 25 uur na toevoeging van kompleet medium te stijgen.

De veranderingen in DNase specifieke aktiviteiten werden gecorreleerd aan de veranderingen in DNA en RNA gehalten ten opzichte van het eiwitgehalte in de schudculturen tijdens de verschillende fysiologische omstandigheden. Er werden verbanden gezocht met de afbraak van vreemd nucleïnezuur, dat in de cel aanwezig kan zijn, o.a. door fagocytose, en met de DNA en RNA synthese. Eensluidende conclusies konden echter, gebaseerd op deze gegevens, niet getrokken worden (hoofdstuk VI).

Getracht werd DNase aktiviteiten aan te tonen in kernen geïsoleerd uit microplasmodia uit Physarum polycephalum. Hieraan voorafgaande werd echter de kernisolatie-methode kritisch bestudeerd en vergeleken met andere kernisolatie-methoden. Het bleek dat de reeds gepubliceerde isolatieprocedure van Mohberg en Rusch (1971) de beste kernpreparaten opleverde, althans op basis van microscopische beoordelingen van deze preparaten met behulp van fase contrast microscopie. Een isolatiemedium geheel bestaand uit zouten was echter in bepaalde gevallen te verkiezen boven het suikermedium van Mohberg en Rusch. Zeker kon het zoutmedium als alternatief voor het suikermedium funktioneren ter uitsluiting van mogelijke artefacten, die door een bepaald isolatiemedium veroorzaakt kunnen worden (hoofdstuk VII).

Hoewel het mogelijk was DNase aktiviteit aan te tonen in kernpreparaten van Physarum polycephalum met een optimale aktiviteit bij pH 4,0, bleken deze aktiviteiten van experiment tot experiment te verschillen. De specifieke aktiviteiten van de DNase aktiviteiten in ruwe enzymextracten was 5 - 50 maal hoger dan in de kernpreparaten en de associatie van zure fosfatase aktiviteit was in de kernpreparaten hoger dan de associatie van de DNase aktiviteit in deze preparaten. Vermoedelijk werd de DNase aktiviteit, die gemeten werd in de kernpreparaten, voornamelijk veroorzaakt door verontreiniging van deze preparaten met cytoplasmatische DNasen (hoofdstuk VIII).

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Curriculum vitae.

De schrijver werd geboren op 20 september 1945 en behaalde, na de lagere school en de middelbare school - St. Dominicus College te Nijmegen - doorlopen te hebben, in juni 1962 het HBS-B diploma. Daarna begon hij in september 1962 aan de Katholieke Universiteit te Nijmegen zijn biologie studie. In maart 1966 behaalde hij het kandidaatsexamen (cum laude) en in mei 1969 het doctoraal examen (cum laude).

Gedurende zijn doctoraal studie bewerkte hij een bijvak zoölogie op op het laboratorium voor Medische Biologie (hoofd: Dr. W.J. van Dongen) o.l.v. Dr. Th.J. Benraad, betreffende de corticosteronsynthese van ratten- en caviabijnieren in vitro. Een tweede bijvak werd gedaan op het Botanisch Laboratorium (hoofd: Prof.Dr. H.F. Linskens) o.l.v. Dr.Ir. J.F.G.M. Wintermans, betreffende de dichloorphenol-indophenolreductie door chloroplasten. Het hoofdvak Chemische Cytologie betrof een onderzoek naar de histonsynthese in kalfslevercellen in vitro o.l.v. Prof. Dr. Ch.M.A. Kuyper op het laboratorium voor Chemische Cytologie.

In het cursusjaar 1968-1969 was hij verbonden als leraar aan de HAVO Notre Dame des Anges te Ubbergen. Van augustus 1969 tot april 1974 werkte hij als wetenschappelijk medewerker aan het laboratorium voor Chemische Cytologie o.l.v. Prof.Dr. Ch.M.A. Kuyper.

S T E L L I N G E N

I

Het is twijfelachtig of de kriteria voor de zuiverheid van chromatine-preparaten, die opgesteld zijn door Bonner c.s. inderdaad iets zeggen over de zuiverheid van geïsoleerd chromatine.

Bonner, J. et al. (1968) Meth.Enzymol. 12B ,3-64

II

De mening van Helmsing,dat de eiwitsynthese in kernpreparaten van Drosophila hydei niet veroorzaakt zou worden door cytoplasmatische verontreinigingen, berust op te weinig gegevens.

Helmsing, P.J. (1970) Biochim.Biophys.Acta 224, 579-587.

III

Uit de experimenten van Chet et al. kan niet geconcludeerd worden, dat de RNase activiteit in spherulerende microplasmodia van Physarum polycephalum niet gecontroleerd wordt door de RNA synthese.

Chet, I., Retig, N. en Henis, Y. (1973) Biochim. Biophys.Acta 294, 343-347.

IV

Er is geen reden om aan te nemen dat er twee verschillende alkalische RNasen aanwezig zijn in kippelevers.

Sarker, N.K. (1972) Currents Mod.Biol. 5, 25-29.

V

De acellulaire slijmzwam Physarum polycephalum is een zeer geschikt object voor het bestuderen van diverse cellulaire processen.

VI

Het is wenselijk, dat de medewerkers van een laboratorium meer inzicht krijgen in het functioneren van taakgerichte groepen.

VII

De schadeloosstelling van verkeersslachtoffers dient, los van iedere schuldvraag, vergoed te worden via een collectieve verzekering van alle verkeersdeelnemers.

VIII

Het verdient aanbeveling functionarissen, die betrokken zijn bij de planning van, en die beslissingsbevoegdheid bezitten bij, de aanleg van wegen in steden, te verplichten enkele dagen per week niet van een personenauto gebruik te maken in hun woon-werk verkeer.

IX

De afwezigheid van de mogelijkheid dat popmuziek musici door de overheid worden gesubsidieerd, zoals dat het geval is voor klassieke muziek musici, duidt op een enge cultuurvisie bij de overheid.

X

Gezien de huidige milieuopvattingen verdient het spreekwoord "beter één vogel in de hand dan tien in de lucht" herziening.

B.J.J. Polman
Nijmegen, 24 juni 1974